

Alarm, but little action, on population

Sooner or later, governments whose populations are still growing quickly will find themselves exposed to external pressures, on the principle that the world's growth is of general concern.

WHAT follows is automatic writing of a kind. The argument last week (*Nature* 362, 275; 1993) that the problem of nuclear proliferation will eventually have to be dealt with by coercion, whatever infringement of sovereignty that may entail, also applies to population growth. In both cases, the violators of good order — those who make bombs (the Republic of South Africa tipped its hand last week — see page 384) and those who let their populations grow without restraint — are a threat to what are called the global commons. The rest of us will be less secure, or less well-off, if they continue in their wayward courses. The big difference is that the international community seems readier to deal with bomb-makers (witness Iraq) than with those whose populations are growing quickly. But for how much longer?

Not very long. Indeed, coercion of a kind has already begun, but passively. It consists of neglect. Thus many African countries have evidently fallen to the bottom of the priority lists of those who administer official technical assistance, while international corporations do not regard them as places where investments prosper. There are good reasons for these implicit decisions: potentially recipient governments are incompetent, corrupt or overwhelmed by civil strife. But the consequence is that the poor become poorer, that population growth continues unabated and that the populations of such countries provide horrific demonstrations of the Malthusian prediction that populations are intrinsically self-limiting; birth and death rates will be balanced by famine and disease. That is why, every year or so, the rich countries are afflicted by waves of compassion for Ethiopia, Somalia and the Sudan, as the children of the famines appear on television.

Population growth is the starting-point for *Preparing for the Twenty-first Century*, a gloomy tract by Paul Kennedy, the Yale historian (HarperCollins, £20.00). It was also the theme of the UN conference on European population at Geneva last week. But those likely to be coerced by others towards slower population growth do not relish the prospect; otherwise, they would not have fought so hard to keep the topic off last year's survey of environmental problems at Rio de Janeiro. Nor is it clear what form of coercion would be both effective and acceptable. One difficulty is that a moderate degree of prosperity appears to be a necessary (but not a sufficient) condition for population stability. (Others include education and a substantial expectation of life at birth.) The danger in policies, explicit or otherwise, that starve quickly growing countries of financial assistance is that they

are then denied the chance of ever qualifying.

Kennedy points out that Malthus's predictions, aimed as they were at eighteenth-century Britain, could have been fulfilled if it had not been for technology, in the form of the Industrial Revolution. With that, of course, came formal education for all and the emergence of groups of professional people able to exploit existing techniques and to develop new ones. Even so, the British population increased fourfold in a century before its growth became less rapid and, eventually, stable. Most of Africa is a long way away from that state of grace, although other population black-spots, until quite recently South America and South-East Asia for example, have demonstrated that progress towards the classical demographic transition is still possible. Even in India, the fertility rate has fallen by a third in the past two decades.

What, in the circumstances, can be done? The first need is that the nature and magnitude of the problem should be openly acknowledged. It is not so much that the world population of 11 billion some expect by 2050 would be unable to feed itself, but that a seemingly transition from here to there will not be smoothly and safely accomplished. The pressure of poor countries on the rich world surrounding them has so far consisted largely of migration, but is it reasonable to expect that growing poverty and growing prosperity can indefinitely and peacefully coexist?

But coercion, in this case, is not easily made effective and acceptable. Seeking to make overseas assistance dependent on demographic statistics would be neither. In any case, economic assistance is needed here and now, but demography changes only slowly. But requiring that recipient governments should commit themselves to externally monitored programmes of mass education and public health would at least create the conditions in which the demographic transition to stability could be approached. If, in return, the rich world agreed that exports from poor countries should be unfettered by trade restraints, there would be a chance that the poor countries would be poor no longer. □

Assessment works

Tokyo University has done well to embark on external assessment of its physics department.

THE physics department at the University of Tokyo is winning a reputation for academic innovation, even daring.



Its decision last year to ask a committee including several distinguished people from overseas to investigate its quality "and to report", at least in part in public, is without precedent in Japan (see page 387). Of course, Tokyo has a high reputation, especially in physics. Professor Akito Arima, who retires this week as vice-chancellor at Tokyo, may have calculated that an inquiry concentrating on this department would prompt conclusions that could only be flattering to the university as a whole, but which would support a cause he has championed for many years — that of persuading the Ministry of Education that even this outstanding academic unit is scandalously housed and poorly equipped.

Predictably but unavoidably, the visiting committee has roundly condemned the physical facilities in the physics department. Just as predictably, it has praised the academic distinction acquired internationally by a large proportion of the department's faculty as well as the quality of the students, both undergraduate and graduate. The question it does not raise is why the Ministry of Education has not yet seen fit (but a new building is being planned) to provide acceptable facilities for a department that would bring honour to any university in the world. The explanation, of course, is the cult of egalitarianism, on this evidence still as strong in Japan as anywhere.

But Arima may have got more than he bargained for in what the committee has to say about the educational pattern found in the physics department. There are neither women nor foreign scientists among the tenured staff. Communications among the ten research groups into which the faculty is organized are much less than good. Students, whether undergraduates or graduates, are too rigidly constrained by the curriculum and by the way in which attachment to a professor is considered irrevocable. Postdoctoral fellows are poorly used, more as dogsbodies for the professor to whom they are attached than as independent investigators, and the same is true of all but the full professors of physics at Tokyo. The committee is on sure ground in asking that these deficiencies should be remedied. Quite what the university will make of the recommendation that there should be regular student evaluation of the effectiveness of individual teachers is another matter; traditional politeness may inhibit even anonymous assessments.

The committee might also usefully have strayed beyond its terms of reference by asking questions about the links between the physics department and the research institutes with which it is associated. They are at present too loose. And those who have already said that the committee overlooked the problem of technical assistance in research are surely correct: do Japanese researchers need the skill in glass-blowing or soldering that circumstances now require of them? Nevertheless, this first venture in external assessment has been a great success. Arima's successor will be able to make good use of it in dealing with a ministry wedded to equal shares for all. He may also use it to good effect internally. Will universities elsewhere (outside enlightened Scandinavia) now follow suit? □

Religious study

The University of Cambridge should have thought harder before accepting a donation joining science and theology.

If further evidence were needed of the lengths to which British universities will go in order to attract endowments from the private sector, it is provided by the announcement two weeks ago by the University of Cambridge of the establishment of a lectureship in Theology and Natural Science thanks to a £1 million donation from best-selling author Susan Howatch. Frequenters of airport bookstands will know of Howatch as the author of blockbusters such as *Penmarric* and the promisingly Dawkinsesque-sounding *Wheel of Fortune*, but it is here, sadly, that her scientific credentials expire. Having realized in 1980 that fame and riches had left her unsatisfied, Howatch decided to follow Cassius Clay and Elvis down the well-trodden road to celebrity revelation.

After six years away from authorship and a school-leaver's certificate in religious studies, Howatch stormed the literary world afresh with a hugely successful novel *Glittering Images*, a story of clerical life in the fictional English town of Starbridge. To everyone's surprise, the novel brought in as much money as her previous secular efforts. Hence the Starbridge Lectureship, welcomed by the university for coming "at a time when the interaction between science and theology can offer much in the search for greater understanding of our current existence". A happy coincidence indeed.

According to Howatch, "science and theology are no longer seen as opposed but complementary, two aspects of one truth". That can be true only in the most superficial sense, that in which some people see one truth and not the other, or vice versa. For the many people who take the scientific and the theological (or at least the religious) view together, it is more common to reconcile the inevitable intellectual conflict on matters such as the after-life by supposing that there are two truths, not two aspects of one truth, or otherwise to suppose that Bible-stories or their equivalents in other religions have an allegorical function of great moral value to believers. There is of course a rich vein of research on the psychology of religious belief, but that is not what Howatch and the university have in mind. What other academic purpose can there be?

It would be churlish to chide Ms Howatch for polluting British universities with the profits of her blockbusters, or even for asking that her money be spent on a venture so empty as the Starbridge Lectureship, but is it proper that one of Britain's leading centres of learning should put a price on its academic rigour and accept her donation? It is not as if the past few years have not seen some happy collaborations between academic institutions and the private sector — most notably the efforts of George Soros in what used to be the communist bloc. Universities everywhere need private funds, but with no strings attached. They should say so clearly or be more persuasive of their academic goals. □

Japanese stimulus package includes billions for computers

Tokyo. Many of Japan's universities and government research institutes are likely to receive new computer facilities thanks in part to a political scandal involving charges of tax evasion against Shin Kanemaru, a former top leader of the ruling Liberal Democratic Party (LDP).

Bureaucrats in Japan's science-related ministries and government agencies this week are scrambling to grab a large slice of a ¥14,000 billion (US\$120 billion) supplementary budget that the government hopes will pull Japan out of recession. Normally, the chief beneficiaries of such one-time supplementary budgets are the Ministry of Construction and construction companies, which receive lucrative public-works projects. But Kanemaru and other LDP politicians under his influence, for example former prime minister Noboru Takeshita, play an important role in directing government money to such public works, and their weakened position has created a unique opportunity to divert money elsewhere.

The Ministry of Education, Science and Culture (MESC) is expected to request about ¥1,000 billion (\$8.7 billion), a large portion of which is for supercomputers, local and wide area networks to link computer facilities in universities and university-related institutes and personal computers in elementary schools. Similarly, the Science and Technology Agency (STA) and the Ministry of International Trade and Industry (MITI) together hope to receive several hundred billion yen, much of it for supercomputers and local area networks. These ambitious requests contrast with just over ¥100 billion allocated for science in last year's supplementary budget of ¥10,700 billion (see *Nature* 360, 8; 1992).

The three government organizations together are asking for about 12 supercomputers and a similar number of mini-supercomputers, far more than the three or four supercomputers the government has been buying each year. MITI hopes to replace an old Cray supercomputer at its supercomputer centre in Tsukuba science city and is also requesting a mini-supercomputer for its Atom Technology Project (see *Nature* 362, 279; 1993) and a supercomputer for its Real World Computing project, both of which will be based in the science city. The plan also calls for an upgrade of the local area network that connects computers in the ministry's Tsukuba institutes and for new computer facilities at its regional research institutes elsewhere.

Similarly, STA hopes to improve com-

puter facilities at its Institute of Physical and Chemical Research (RIKEN) near Tokyo and at other agency institutes. And MESC has even more ambitious plans to buy a large number of mini-supercomputers and supercomputers for Japan's universities and



These MITI institutes hope to share the wealth.

to network them together.

When informal negotiations for the supplementary budget began a few weeks ago, MESC, MITI and STA as well as the Ministry of Posts and Telecommunications hoped to request funds for a high-capacity national backbone network to link computer facilities at government research organizations throughout Japan. But each ministry and agency had different ideas about the backbone network, delaying action until fiscal 1994, which begins on 1 April 1994.

Tateo Arimoto, director of STA's science and technology information division, says that such computer network infrastructure has until now been a "low priority" but

that the supplementary budget has provided a "big chance" for change. Although he expects the Ministry of Finance to reduce the requests from the three government organizations, he is confident that the move to improve infrastructure "will continue".

Another factor driving the Japanese government's sudden enthusiasm for high-capacity computer networks is the proposed gigabit national computer network in the United States. Present plans in Japan call for only a six-megabit backbone, considerably less than existing US systems but the most that can be handled by the fibre-optics system of the domestic telecommunications company Nippon Telegraph and Telephone (NTT). The supplementary budget is also expected to contain money to increase the capacity of NTT's fibre-optics network.

Arimoto says that "open bidding" will be held for the new computer systems, and the president of the Japanese subsidiary of the US supercomputer manufacturer Cray Research was reported to have said that he expects Cray to get "about a third" of the supercomputer orders under a 1990 US-Japan agreement on supercomputer trade that calls for fair and open procurement practices. But it is not clear if these rules will apply to the purchase of the many smaller computers.

The supplementary budget is being compiled in time for Japanese Prime Minister Kiichi Miyazawa to discuss parts of it with US President Bill Clinton when he visits Washington in mid-April. Clinton is expected to put pressure on Japan to buy US computers to reduce a large trade imbalance.

David Swinbanks

German education summit planned

Munich. The long-awaited but often delayed education summit on improving the efficiency of Germany's universities, first proposed a year ago by Chancellor Helmut Kohl (see *Nature* 358, 361; 1992), will probably take place in September. The impetus for the meeting is the strain of chronic overcrowding, the result of a law that guarantees a place to every student who passes an examination at the end of high school and the fact that students spend too long completing their courses (the average age of a graduate is 28).

Delays in fixing a date have in part been caused by disagreements over whether the reforms should in principle be financed by

new money. The government has refused to attend a summit where money is on the agenda, insisting that the universities should first put their house in order. It also wants to see more evaluation of research carried out in universities. On the other side, the universities have ruled out preparing for a summit where the possibility of extra financing is excluded.

The presidents of Germany's 16 states last week agreed to negotiate next month with Kohl to break the deadlock. The most likely date is in the autumn; any further delays will land it unhappily in Germany's preelection period.

Alison Abbott

NSF does well, NIH does not in Clinton's 1994 budget

Washington. The US budget for fiscal year 1994 that President Bill Clinton will next week send to Congress assigns a favoured position to the US National Science Foundation (NSF) in the projected economic recovery while treating the US National Institutes of Health (NIH) as a bit player, reinforcing the perception that the president's vision for technological change does not include biomedical research.

The president is requesting \$216 million more in the year beginning on 1 October for NSF, an increase of 7.3 per cent that would raise its budget to \$3.18 billion. This week the US Congress was expected to approve a \$16-billion supplementary budget package for 1993 that would add \$206 million to NSF's existing budget. Even so, a combination of those two increases would raise NSF only slightly above the level requested last year by former President George Bush but rejected by the US Congress.

In contrast, unofficial budget figures for NIH show an increase of only 3.3 per cent (\$340 million) that would bring its budget

to \$10.67 billion. The increases are almost entirely in three areas — breast cancer research, AIDS and the human genome project — previously targeted for growth by the Congress and the administration, leaving nine of the 18 institutes and centres with less money next year than at present.

This has been an unusual year for the president's budget, normally submitted to Congress at the end of January. New rules allowed Bush to compile only an outline of the annual \$1,500 billion federal budget; despite working feverishly for several weeks, the new administration has pushed back from mid-March to early April the release of its budget. Congress has begun work on the budget anyway, with NSF and NIH officials testifying last week at separate appropriations hearings. NSF provided details the next day, but NIH is officially remaining silent until the full budget is unveiled sometime next week.

For NSF, the good news in the president's request is an increase of 7 per cent for its basic research programmes, 14 per cent

more for science and mathematics education and enough to keep three major new facilities on schedule — \$17 million towards twin 8-metre telescopes to be built jointly with Britain, Canada and three Latin American countries, \$43 million towards a laser interferometer gravity wave observatory (LIGO) and \$12 million towards a national high magnetic field magnet laboratory. The bad news is that Congress, which jealously guards its role in setting spending priorities, may remember NSF's good fortune this year in getting a supplementary budget that provides about half of the increase it had originally sought from Congress.

"If this committee had had an additional \$206 million available to it last year, it probably would not have given it all to the NSF", said Representative Louis Stokes (Democrat, Ohio), chairman of the House appropriations subcommittee that funds NSF and several other agencies. "And if this subcommittee's allocation does not match the president's 1994 request, we'll have to take into account the rather generous increase given to NSF in the 1993 supplemental."

In contrast, there is little for NIH to cheer about in what Clinton has so far proposed. The supplementary appropriation contains only \$9 million for its share in a high-speed computer initiative, and the 1994 request, if

NIH plans to begin AIDS drug trials at earlier stage

Washington. The US National Institutes of Health (NIH) will begin treating people who are HIV-positive as soon as possible after infection in the wake of new information (see *Nature* 362, 355; 1993) that the HIV virus is active in the body in large numbers much earlier than was previously thought.

"We must address the question of how to treat people as early as we possibly can with drugs that are safe enough to give people for years and that will get around microbial resistance", says Anthony Fauci, director of the US National Institute of Allergy and Infectious Diseases (NIAID). Any delay, he adds, would be the result of questions regarding safety and resistance rather than a lack of funds.

However Fauci, who co-authored one of the two papers on the subject published last week (*Nature* 362, 355 & 359; 1993), hotly denies the suggestion by one of his co-authors, Cecil Fox, that the new evidence means that "\$1 billion spent on vaccine trials" has been "a waste of time and money" because the trials were started too long after the patients were infected and were ended too quickly. Fox, of the Yale University School of Medicine, says that data showing the extent to which the HIV-1 virus is active in the lymphoid organs during the early stages of HIV infection mean that "for the first time, the pathogenetic dynamics of the disease are

properly understood".

But Howard Temin of the University of Wisconsin is more cautious. "I only wish life were so simple", he says. "If we understood its pathogenesis we'd understand how the HIV virus does what it does — but that mystery has not dissipated."



No meeting of minds for Fauci, left, and Duesberg.

John Tew of the Medical College of Virginia in Richmond says that the new evidence makes a strong case for early treatment of HIV-positive patients. "These data would argue that early intervention is critical and, if they are correct, that very early intervention may have a heck of a lot of benefits."

AIDS activists welcomed the new information but said that the scientific community has been slow to understand the significance of infection of the lymph tissue.

"We've known about this for five years, but we're glad it is now in the public domain", says Jesse Dobson of the California-based Project Inform. Dobson says that he hopes the work would lead researchers to take "a more pathogenesis-focused approach, as opposed to a drugs-based approach".

The authors of the papers and their supporters see the evidence of infected lymphoid tissue as a mixed blessing in treating HIV-positive patients before AIDS develops. As Ashley Haase of the University of Minnesota and co-author of one paper, puts it: "It is very sobering that we have infection early on, on such a vast scale. But I'm impressed by how the human immune system is able to contain the infection for a very long time."

Critics of the conventional view of HIV as the precursor of AIDS say that there is nothing in the papers to change their minds. Peter Duesberg of the University of California at Berkeley says that the data are "absolutely irrelevant" to AIDS. "We are several paradoxes away from an explanation of AIDS — even if these papers are right", says Duesberg, who believes that AIDS is independent of HIV and is a result of recreational drug abuse in the West and immunization programmes in the developing world.

Colin Macilwain

funded, would mark the second year in a row in which the NIH budget has failed to keep pace with inflation. An increase of \$200 million for the National Cancer Institute is actually a continuation of a programme begun this year by the US Army on breast cancer research, and an increase of \$28 million for the National Center for Human Genome Research reflects a promise made by NIH director Bernadine Healy to its incoming director, Francis Collins, to set up a laboratory on the NIH campus. The National Institute for Allergy and Infectious Diseases would receive an increase of \$86 million for AIDS research. The budgets for the rest of the institutes would be flat, with actual decreases for the heart, lung and blood, ageing, alcohol abuse, arthritis, deafness, diabetes, eye, mental health and neurology institutes.

This year, for the first time, NIH has divided the budgets of each institute into three parts that correspond to its long-awaited strategic plan. Those categories — critical health needs, critical technologies and intellectual capital — are intended to demonstrate the role that NIH plays in meeting important national goals — improving public health, creating new technologies and training the next generation of scientists. But so far that message, repeated endlessly by Healy, has not been translated into more money.

"It's a devastating proposal", says David Moore of the Association of American Medical Colleges about Clinton's 1994 budget. "We have failed dramatically in getting our message through [about the value of biomedical research] to the new administration, and I don't think that Congress is going to bail us out this year. It shows how much work we have to do on the [fiscal year] 1995 budget."

Jeffrey Mervis

Britain funds taxonomy studies

London. A year after a committee of the British House of Lords criticized the government's neglect of taxonomy (see *Nature* 355, 488; 1992), the Natural Environment Research Council has announced that it will spend £2.5 million (US\$3.5 million) over the next five years.

Those selected to receive the funding, chosen from a pool of 19 applicants, are the University of Glasgow, the Imperial College of Science, Technology and Medicine and the University of Reading. Each will receive funding for two research fellowships, postgraduate training and research carried out with local museums.

Council officials point out that the study of taxonomy has been stimulated by recent developments in genetics and molecular biology. Its importance has been highlighted by debates on biodiversity.

David Dickson

Britain to help coal centre develop cleaner technologies

London. The British government is to provide an extra £4 million (US\$6 million) a year for the next three years to support research into cleaner and more efficient methods of burning coal. The new money was announced last week by the Department of Trade and Industry (DTI) as part of a White Paper (policy document) on energy policy that proposes closure of as many as 30 coal mines in preparation for the privatization of

However, in contrast to other coal-producing countries, Britain has been reluctant to provide public funds for plants to demonstrate the economic feasibility of its techniques. Spain has recently agreed to cover 60 per cent of the costs of a new demonstration plant using integrated gasification combined cycle (IGCC) technology largely developed in Germany, and several IGCC plants are planned in the United States with \$2 billion over several years from the Department of Energy's Clean Coal Technology Programme.

The White Paper reinforces Britain's stance by advising against a request from industry that the government provide as much as £200 million for a 300 MW demonstration gasification plant. Such a public investment "would not be justified" at present, it concludes, adding that "international suppliers" can provide the technology when and if it is needed.

The decision to provide extra money for research and development into the topping cycle follows a report from a working party of industrial-

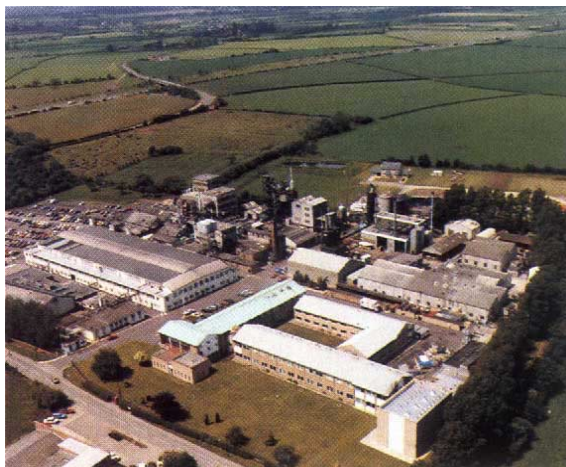
ists and academics to the DTI that said the cycle offers major advantages over most competing systems in generating efficiency. But it also said that both the overall system and its components needed further development before they could be offered commercially.

In response, the government has now agreed that an industry-led consortium should take responsibility for the topping-cycle programme now carried out by British Coal and that it, together with British Coal and the European Commission, will continue to fund research into component development at the CRE.

But the government's announcement has done little to allay fears that the privatization of the coal industry will lead to a major reduction in funding. Critics point out, for example, that the successor companies to the Central Electricity Generating Board, privatized in the 1980s, have reduced spending on research by half.

Government officials claim that the new money is designed to prevent this from happening. But others remain unconvinced and point out that the DTI's clean-coal research budget of less than £5 million pales next to the \$600 million spent last year in the United States in 1992 and is only one-tenth that of Germany.

David Dickson



Grant staves off closure for CRE.

the coal industry. Much of it will be used to support development of the so-called 'topping cycle', a technique for burning coal pioneered in Britain that combines fluidized bed combustion and gasification techniques.

The extra funding has lifted a cloud over the future of the Coal Research Establishment (CRE) at Stoke Orchard in Gloucestershire. CRE will become an independent research centre when its parent body, British Coal Corporation, is privatized later this year. Without the support of DTI during the transition, the research centre — already suffering from low morale because of funding cuts in recent years — would have faced redundancies among scientific staff and possibly closure. But the extra money is being seen as "too little, too late" by those who believe that Britain has lost out to the United States, Japan and Germany in a race to produce environmentally acceptable coal-burning technology in the next century.

Researchers at CRE have been at the forefront of efforts to develop various advanced clean-coal burning techniques. One is fluidized bed combustion, in which a stream of air from the bottom of a combustion chamber 'fluidizes' a mixture of coal and crushed limestone. A second is the gasification of coal by using steam and either oxygen or air.

Panel criticizes South African advisory councils, priorities

Cape Town. South Africa spends too much on nuclear energy and high-energy physics, according to an outside review of government funding of science that was also very critical of the actions of two research advisory councils.

The review, by the International Development Research Centre of Canada, followed the approach taken by the Committee on Science and Technology Policy of the Organization of Economic Cooperation and Development (OECD) and was led by the former chairman of the OECD committee, James Mullin. Mullin was accompanied by Deanna Ashley of the Jamaican Ministry of Health; Thomas Odhiambo, director of the International Centre of Insect Physiology and Ecology in Nairobi; Lydia Makhubu, vice-chancellor of the University of Swaziland; and Geoffrey Oldham, former director of the Science Policy Unit at the University of Sussex.

Responding to an invitation from the African National Congress (ANC), the panel visited South Africa for two weeks last autumn. Its members found themselves unable to judge the value of the Scientific Advisory Council (SAC), which advises the cabinet on the allocation of the science budget, because its recommendations to government are kept confidential. Even so, the SAC was criticized for not having raised questions about the effects of a five-year decline of 25 per cent in the purchasing power of grants to the statutory councils after its chairman, Chris Garbers, informed the mission that all the SAC's advice has so far been accepted by the government.

Criticism was also directed at the National Accelerator Centre (NAC), which is administered by the Foundation for Research Development (FRD), which funds basic academic research. The centre accounts for a quarter of the foundation's annual budget of \$42 million, although this money is earmarked by the cabinet. The major facility at NAC is a variable energy cyclotron capable of producing 200 MeV protons (see *Nature* 327, 271; 1987).

One-third of its beam time is allocated to research in nuclear physics, where novel work is limited by the energy range produced by the beam. The remaining beam time is used for neutron and proton therapy for cancer and for medical isotope production. But the review concluded that the medical justifications for "the continued funding of NAC do not withstand scrutiny from any reasonable public health perspective, given the costs involved".

Also under fire is the Atomic Energy Corporation (AEC); its annual state subsidy of \$142 million compares with the \$238

million given to the country's six research councils. The AEC's main function is to produce enriched uranium for South Africa's sole nuclear power plant, although it derives some income from non-fuel products as well, and spends about \$25 million annually on research related to these functions.

The review criticizes the AEC's development as having "ignored entirely any factors relating to the commercial viability of processes being developed". It suggests that the knowledge acquired in the course of the AEC's development be redeployed in non-nuclear industrial activities. The corporation's chief executive, Waldo Stumpf, agrees that the government's support has been generous but points out that the proportion of its budget derived from the state subsidy is expected to decline from two-thirds to one-fifth in five years.

The mission aimed its harshest criticism at the Human Sciences Research Council (HSRC), which carries out in-house research. Its usefulness, according to the review panel,

is "irretrievably compromised [because of its role] as the source of much of the analysis that lay behind the policy of Grand Apartheid". The panel suggests that government departments could commission social-science research projects as needed.

The report has been generally well-received by universities, despite some concern about its perceived bias against fundamental research. Although the panel praises fundamental science, which currently receives less than ten per cent of what the government spends on civilian research and development, it suggests that the two major agencies in this area, the FRD and the Medical Research Council, should allocate more of their funds to special and public health programmes respectively. In particular, the mission believes that agricultural research receives too much money and that environmental research should receive much more.

The report urges a prompt and thorough review of the directions of military and space research. While this fell within the mission's terms of reference, Mullin says that it was denied access to relevant information. Although Mullin says that "debate now has to progress from an analysis of present deficiencies to suggestions about possible solutions", the panel decided that the decisions must be left to the scientific community itself.

Michael Cherry

South Africa admits to having six bombs

Cape Town. The South African president, F.W. de Klerk, ended twenty years of speculation about the country's nuclear capability when he announced in parliament last week that six nuclear devices had been constructed — but never tested — during the 1980s. Since South Africa acceded to the Nuclear Non-Proliferation Treaty (NPT) in July 1991, the devices (which had been constructed by the quasi-governmental armaments corporation ARMSCOR) have been dismantled and the highly enriched uranium inside them has been returned to its source, the Atomic Energy Corporation (AEC).

The AEC will use this material for the commercial production of isotopes at its SAFARI-1 research reactor, built by the United States in 1965 and operating under international safeguards. That is a plausible explanation, given that the reactor was designed to use uranium enriched to 93 per cent, although it has been operating on 45 per cent loads since the United States suspended shipments in 1977.

South Africa's pilot enrichment plant at Valindaba (a word from the African language Sotho, meaning "about this we do not speak"), near Pretoria, became fully operational the following year, and in 1981 it was announced that it had produced 45 per cent reloads for SAFARI-1. However, between 1978 and early 1990, when it was decommissioned (see *Nature* 347, 417; 1990), the pilot plant also produced enough material enriched to weapons grade (usually 93 per cent) to produce seven nuclear devices, six of which had been built by the time de Klerk was elected in September 1989.

Upon assuming office, de Klerk halted production of weapons-grade material and ordered the dismantling of the devices and the return of the enriched uranium to the AEC. The AEC's state subsidy, which peaked at R980 million (US\$310 million) in 1989–90, has been reduced by more than half in the past three years and no longer includes expenses associated with enrichment for weapons.

South Africa included the weapons-grade uranium in its inventory submitted to the International Atomic Energy Agency (IAEA), which has carried out more than a hundred inspections since October 1991. The IAEA has been careful to honour its commitment to keep confidential what it has learned, but there is scepticism that South Africa has disclosed all its weapons-grade uranium.

De Klerk emphasized that the bombs were never tested, a claim substantiated by the IAEA's failure to detect evidence of fallout. He claims that the devices, although designed for delivery from an aircraft, were to be used only as a deterrent.

M.C.

SSC detector collaborators shun financial commitment

Washington, London & Japan. US scientists responsible for building one of the two large detectors for the Superconducting Super Collider (SSC) in Texas are counting on money from other countries that is unlikely to materialize, according to government officials in Europe, North America and Asia. At the same time, the fate of the \$10-billion proton-proton accelerator appears to rest with President Bill Clinton's willingness to persuade a reluctant US Congress to continue its funding.

The Solenoid Detector Collaboration (SDC) is a \$589-million project involving the United States and seven other countries — Britain, Canada, China, France, Italy, Japan and Russia (see *Nature* 357, 102; 1992). The United States is expecting those countries to finance nearly 40 per cent — some \$231 million — of the detector, which contains a vast array of equipment and electronics needed to record and interpret the results of hundreds of millions of collisions a second. At present, SSC documents refer only to "expected participation" by other countries at a level equal to what it would cost to build a particular piece of equipment in the United States (see table).

None of the seven countries, each of which faces the same budgetary pressures that have led the United States to seek outside support, has made a commitment to pay its share. But SSC officials, ever optimistic, expect each team of collaborating scientists to submit a request to the appropriate funding agency in its country and to win approval over the next 12–18 months to keep on schedule for completion in 1999.

But that seems unlikely to happen. At best, individual scientists now collaborating with the SSC laboratory on generic detector research say that they may at some point ask for money, but none is confident of success.

Most European countries, scheduled for contributions of roughly \$15 million, support a similar machine — the Large Hadron Collider (LHC) — still in the planning stages at CERN, the European Laboratory for Particle Physics in Geneva. The Japanese,

expected to contribute half — \$116 million — of the overall foreign share, have repeatedly avoided any commitment to the SSC or the SDC. A US-Japanese working group set up last year has not met since the US presidential election, although the issue is expected to be raised later this month when Prime Minister Kiichi Miyazawa meets Clinton in Washington.

The Canadians, in contrast, strongly

ing 10 per cent of Britain's potential contribution to the LHC". But no request has been submitted, Cashmore says.

Claude Wolff, French science attaché and organizer of the meeting, says that "I am not aware of any request" for the estimated \$13.7 million and that the French scientists now working on the SDC are being paid by the SSC, not the other way around. An official of the Centre National de la Recherche Scientifique (CNRS) says that the French government would need to triple its research budget to provide "any money at all".

The Canadian team, headed by Bob Ott of the University of Toronto, expects to submit a proposal later this year to fund parts of two pieces of equipment that some

two dozen Canadian physicists want to develop. The group hopes to receive money from three government agencies, in particular the National Science and Engineering Research Council, which has provided C\$1.25 million over two years for preliminary work.

Although the Canadians believe that the SDC is far superior to the LHC, the government will be hard-pressed to find the money. An advisory panel on nuclear and particle physics has been told to

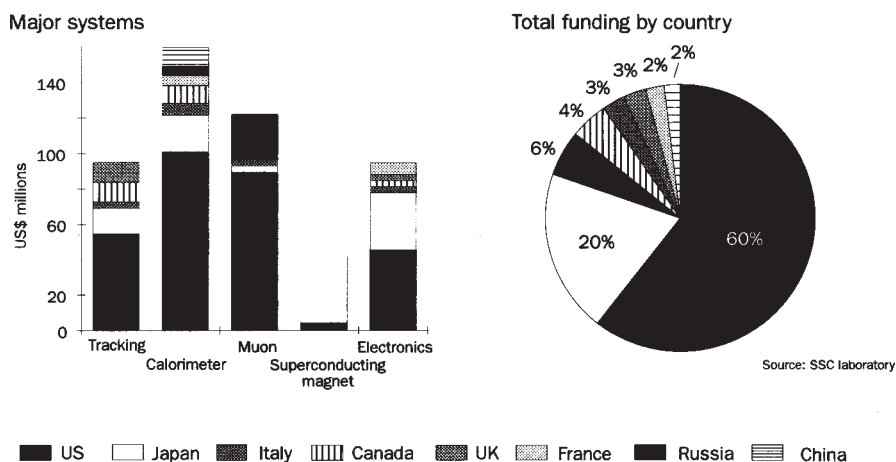
assume a flat budget for the field, which Ott calls "a disaster scenario", but he remains hopeful that some money will be found to continue the US collaboration.

In Japan, a \$12-million programme for collaborative research on a range of US high-energy physics projects includes generic research on the SDC. But Takahiko Kondo of KEK, Japan's National Laboratory for High-Energy Physics, says that there has been no official request for \$116 million and that the figure neither corresponds to actual construction costs in Japan nor includes the infrastructure needed to build the superconducting coil and other important features of the detector.

Meanwhile, the fate of a \$640-million request to the US Congress for the SSC in the fiscal year that begins on 1 October could hinge on the extent of support offered by the president. "If the president puts his full weight behind it, we'll be OK and you'll start to see some of the other countries fall in line", says William Happer, director of the Office of Energy Research at the US Department of Energy. "If not, it's a foregone conclusion that the SSC will be killed."

**Jeffrey Mervis, David Dickson
& David Swinbanks**

Potential global contributions to the SDC



favour the SSC, but the country's annual budget for high-energy physics is less than the \$25 million needed for the SDC. The Russians, expected to contribute \$33 million, welcome foreign contributions to their cash-starved scientific system but have little money to spend. The Chinese contribution of \$10 million, the smallest of all, appears the one most likely to materialize.

Roy Schwitters, director of the SSC laboratory in Texas, discussed the subject earlier this year with more than two dozen foreign science attachés based in Washington. His comments about potential contributions to the SDC appear to have shocked or amused many in his audience.

"I was astonished to hear reports [of what Schwitters said] because there is no commitment to spend any money on the SSC", says Sir Mark Richmond, chairman of the UK Science and Engineering Research Council. "If I had \$15 million to spend in this way, I would spend it on the LHC."

Roger Cashmore, a physicist at the University of Oxford and head of the four British teams involved in the SDC, explains that the \$15 million represents "the maximum viable contribution that would also make sense scientifically while not exceed-

SERC broadens its role in support of industry

London. The UK Science and Engineering Research Council (SERC) is to launch a major effort to support research into new industrial products and manufacturing processes. Known as the Innovative Manufacturing Initiative and run by a committee dominated by industrialists, the new scheme will have an annual budget of about £30 million (US\$45 million) for research to boost Britain's capacity for wealth creation.

The new initiative, planned for later this year, is intended both to clarify the council's role in helping industry and to strengthen its hand in anticipation of the government's forthcoming White Paper (policy document) on science and technology, which is expected to contain a major shake-up in the organization of the council.

SERC would like to separate its support for the research needed to underpin the country's industrial base from those areas of science — such as astronomy and particle physics — pursued primarily for 'cultural' reasons. It has begun to increase its support of industry-orientated research in line with a recent review of its engineering programme that recommended a greater "coincidence of purpose" between the council and industry, even at the expense of university-based research groups. Last week, for example, SERC's chairman, Sir Mark Richmond, told the House of Lords Select

Committee on Science and Technology that the council is debating whether to make grants directly to industrial scientists and not only to those working on a joint project with universities.

Any such move would be widely welcomed by industry itself, which can now apply for direct research funding only from the Department of Trade and Industry (DTI).

But universities are concerned about the proposals. They argue that allocating research grants directly to industrial research groups could undermine SERC's efforts to support the transfer of technology from the academic to the industrial world.

Richmond agrees that the new funding directions would mean reduced support for the science base "in the old-fashioned sense". Indeed, SERC has already set itself the goal of reducing total expenditure on basic research by 10 per cent over the next three years as it shifts the balance between basic and applied research from 45:55 in 1991-92 to a new goal of a 40:60 distribution.

In its corporate plan for this period, being published this week, the council also sets out a number of new developments aimed at strengthening its support for industrially relevant research. In addition to the new multidisciplinary initiative in innovative manufacturing, these will include increased involvement by industry representa-

tives in "top-level matters of broad policy and programme balance" and a 'concordat' with the DTI. Later this year, SERC will begin a 'business awareness scheme' to help academic scientists to understand how their discoveries could be commercialized.

The corporate plan also reveals that the council intends to launch a pilot scheme for part-time PhD awards, in line with the recommendations of its recent report on post-graduate engineering education (see *Nature* 362, 7; 1993). And SERC has decided virtually to double its public relations budget to develop teaching materials for secondary schools and to establish visitors centres at the council's main laboratories.

Given the costs of international subscriptions, the council says it plans to study ways of reducing its contributions to the European Laboratory for Particle Physics (CERN) and the European Space Agency (ESA). According to Richmond, the council is also looking at ways of transferring funds from physics to other fundamental sciences, in particular chemistry and biology.

How much of these plans remain intact after the publication of the White Paper remains to be seen. Richmond doubts that SERC will survive in its present form but is confident that the direction in which he wants to take the council is in line with the government's thinking. **David Dickson**

OTA panel opens inquiry into patenting of genes

Washington. Members of a panel meeting last week for the first time to advise the US Congress on gene patenting and the human genome project believe that action is needed to protect biotechnological innovation but do not agree on what should be done. The 17-member advisory panel, formed to help the congressional Office of Technology Assessment (OTA) prepare a report on the subject, raised the possibility of restricting patents to specific uses of particular gene sequences or imposing restrictions on the time for which patents can protect them.

The OTA report was requested by Senator Edward Kennedy (Democrat, Massachusetts) and Senator Mark Hatfield (Republican, Oregon) after the international fracas that followed a decision in 1991 by the National Institutes of Health (NIH) to apply for patents on thousands of human gene sequences. Hatfield is concerned about the ethical issues raised by claims of intellectual property rights on human genes. Kennedy is thought to be seeking intellectual ammunition to protect the biotechnology industry in his home state.

The advisory panel includes Craig Venter, the former NIH official whose \$70-million Institute for Genomic Research in

Maryland is proceeding with a rapid programme to identify all important human genes. An associated company, Human Genome Science Inc., aims to exploit the institute's findings commercially.

"There is an explosion of new information available", said Venter after the meeting. "The question is whether the existing patent system is set up to cope. There may be the need for a couple of legislative changes, but changing the patent laws is like opening up a Pandora's Box."

This week, Venter will announce at a Washington conference organized by *Nature Genetics* that his institute expects to isolate and sequence "the majority of human genes" in the next 12-18 months. He promises that all information not directly exploited by Human Genome Science will be made available to outside researchers.

Tom Kiley, a California patent lawyer and director of several biotechnology companies, says that patent law already allows for the utility of a given gene to be tightly specified — provided that the patent office has sufficient resource to guard against abuse. "One way forward is to change the law", he says. "Another is to give the patent office the money to do its job properly."

There is the chance that the systematic identification and patenting of human gene sequences, together with the growing commercial involvement of biotechnology companies, will eventually lead to patents being invoked to block the work of rivals. "Congress should address the issue of research exemption in case the patent system is used to discourage innovation", he says.

Bob Cook-Deegan of the Institute of Medicine warned against an international free-for-all in pursuit of the human genome, calling it "an icon for how science is changing. Scientists are making an uncomfortable transition from their former role as knights in shining armour to a new public image as commercial mercenaries."

The OTA study coincides with an investigation by the United Nations Educational, Scientific and Cultural Organization into the human genome project. The NIH patents led to concern, especially in Britain, about the willingness of the United States to share gene-sequence information with researchers elsewhere. OTA included four non-US members on its advisory OTA panel, which will meet twice more before submitting its report in April 1994.

Colin Macilwain

Panel suggests big changes at Japan's leading university

Tokyo. The physics department of Tokyo University, one of Japan's biggest and best research departments, has serious deficiencies in organizational structure and physical facilities despite producing "outstanding" research, according to a committee of distinguished foreign and Japanese scientists that conducted the first external review of a Japanese university department. The committee, in a report released on 30 March, makes a series of recommendations that, if implemented, could have a profound effect on university research throughout Japan.

The review, carried out by the committee during a three-day visit in January (see *Nature* **361**, 196; 1993), is an initiative of Akito Arima, president of Tokyo University, who has pushed hard for reform of Japan's universities. The committee's report was released the day before Arima's retirement and thus its fate lies with his successor, Hiroyuki Yoshikawa. Senior officials at the Ministry of Education, Science and Culture are confident that Yoshikawa will take up Arima's lead.

The physics department, with 35 professors and associate professors in fields including astrophysics, biophysics and non-linear dynamics and fluid mechanics, is already in the forefront of university reform. The department has modified the traditional *koza* system, which divides departments into independent subfields ruled by one professor with an associate professor and two research associates under his command. Instead, professors and associate professors now have equal status, and several research associates have been promoted to associate professor.

Nevertheless, the committee members find "much room for improvement". They note that nearly all of the younger (under 40) department members are either research associates, a tenured position with a mixture of teaching and research responsibilities but with little immediate prospect of promotion or the chance to develop a strong independent research programme, or postdoctoral fellows, who take direction from one professor and have little opportunity to pursue their own research.

The committee recommends that the position of research associate should gradually be converted to a non-tenured assistant professor position with greater responsibilities for research and teaching. It also calls for a large increase in the number of postdoctoral fellows.

The committee also notes the absence of women faculty members and foreign faculty members and recommends a "special effort" to recruit women at every level. "This apparent failure to bring exceptionally talented

physicists, regardless of their sex or country of national origin, to the Physics Department ... means that students and faculty alike are deprived of the opportunity of having daily contact with some of the leading physicists of our time."

The committee recommends that all new faculty appointments should be "widely advertised outside Japan" without requiring candidates to speak Japanese. It suggests that the relatively poor English of the students should be improved by greater exposure to English-speaking faculty and by attendance at international conferences.

Pointing to physical conditions that are "miserable and utterly unacceptable", the committee says that there is an urgent need to create more space and a better working environment even before new buildings are erected. The committee also comments on the lack of communication between different sections of the department and calls for greater interaction through regular colloquia and informal discussions.

The report says the quality of students is among "the very best" in the world (apart from their lack of ability in English). But it points out that the curriculum for undergraduates is too rigid and does not allow talented students to move at their own pace. The committee members also suggest that graduate students should be given greater freedom to change research topics and supervisor, and that students be allowed to evaluate faculty members (a revolutionary idea in Japan).

Robert Geller, a US geophysicist and one of the few permanent foreign faculty members at Tokyo University, says he agrees with the "spirit" of the committee's recommendations but that converting research associates to assistant professors would create a system of "all chiefs and no indians". He believes that a chronic lack of technicians in Japan's universities must first be addressed, however, because associates now fill the role of technicians.

The marginal English skills of students is not just a function of language, according to Geller. "Students are unable to express themselves even in Japanese", he says, adding that the Japanese education system fails to teach students "how to write clear reports, debate and make speeches".

Akiyoshi Wada, a former member of the physics department and former dean of the faculty of science, also points to the committee's failure to highlight the shortage of technicians. But he says the overall report is "excellent" and that "the committee has achieved more in three days than a Japanese government committee would in one year".

David Swinbanks

Indian scientists decry favouritism of research system

New Delhi. A majority of Indian scientists believe that people are promoted on the basis of favouritism rather than on the quality of their work and that criteria such as professional contacts and loyalty to a superior are weighted more heavily than the number and impact of papers published, awards received or patents granted.

This is one of the findings of a survey conducted by the National Institute of Science, Technology and Development Studies (NISTADS) examining the disproportionately low impact on global science of the vast numbers of Indian scientists. Some 1,450 scientists at all levels in universities, government research institutions and industries were asked about working conditions, professional norms and other factors relating to the conduct of science.

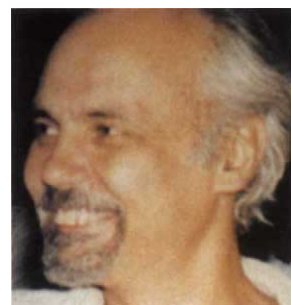
Most of those polled were dissatisfied with the prevailing ethical and professional standards and were particularly unhappy with the way in which research is evaluated, with the reward system and with recruitment and promotion practices. More than 55 per cent believe that knowing the right people plays an important role in obtaining research grants and that institutions have failed to discourage poor performance and to provide the proper research environment.

The survey found that Indian scientists react badly to criticism, hesitate to criticize their colleagues and seldom communicate or interact either with their immediate colleagues or with scientists outside their own institutes. There is a lack of institutional support for mobility and only the most senior scientists have opportunities to travel.

NISTADS found that India's lacklustre performance stems more from poor management of the research system than from a lack of facilities. The authors of the study, published in the February issue of the *Journal of Scientific & Industrial Research* (**52**, 81-94; 1993) conclude that existing policies "are neither conducive for nurturing excellence nor for the promotion of applied science".

K. S. Jayaraman

Correction



A recent article (*Nature* **362**, 198; 1993) about the appointment of Fotis Kafatos as director of the European Molecular Biology Laboratory was accompanied by the wrong photograph. Kafatos appears left.

What goals for UK science?

SIR — After many years as a scientist, I optimistically believe that the sciences have a vital part to play in the improvement of the 'human condition' and, by the way, in the regeneration of the British economy. I take it that this view is shared by the leaders of our profession. It comes as a disappointment, therefore, that all the responses to the Waldegrave initiative expressed by speakers at *Nature's* meeting on 19 March about the forthcoming White Paper on science confined themselves to the straitjacket of questions asked by the minister. Who ever heard a politician naively answer the question asked rather than expressing his or her own views?

One or two members of the audience likened the platform attitude to 'rearranging the deckchairs on the Titanic' and I must concur with that view. Is it too cynical to believe that the spokesmen of our profession are more concerned with the funding of their particular disciplines or institutions than with the wider aspects of the role of the sciences in our present predicament?

Why otherwise would every speaker take it upon himself to become an amateur economist, organizing the division of the research cake, rather than a professional scientist, enthusiastic about the role of science in regeneration and vigorously promoting its share and place in government? The public image of science is not particularly high, and until its most senior representatives defend and promote and, if they believe in it, expand its role, they will get the ministerial representation they deserve, well intentioned but powerless.

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Still second class

SIR — A perceptive letter, "Second class scientists?" (*Nature* 346, 213; 1990), drew attention to a 30–43 per cent differential in salary for incumbents of a position at the University of Auckland according to whether their undergraduate training was medical or scientific. I hesitate to add to this picture, but it is only one aspect of a discrimination against biomedical scientists that is deeply entrenched in New Zealand's oldest and at present moribund medical faculty, that of the University of Otago.

In addition to the salary differential, incumbents with postgraduate qualifications are appointed as senior lecturer if medically trained but as lecturer if scien-

tifically trained: this can be a 10-year seniority differential. Promotion is harder for scientists. A very productive member of my research unit, approved by her grant funding authority for promotion to senior research fellow, was denied promotion by the university. A medically trained colleague of the same age with a less impressive research record had previously been promoted to associate professor.

By persistence, some of us have reached the level of full professor. We do not receive a personal chair like our medically trained colleagues, or as we would in other faculties of the university. Instead, we are designated professorial research fellows, listed in the university calendar at the end of the staff list after lecturers and denied use of the title professor. A recommendation in 1991, by the four medical school deans, that professorial research fellows be given the right to use the title was vetoed by the vice chancellor's office of the University of Otago which controls three of the schools. This unfathomable pettiness was explained as a concern not to devalue the status of existing medical school staff. In truth, research fellows greatly overshadow corresponding staff in professional reputation. Rip van Winkle sleeps on soundly in Dunedin.

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SI units explained

SIR — Two recent articles^{1,2} once more underline an apparent lack of understanding of the *Système International d'Unités* (SI).

The SI is an extension of the metre/kilogram/second/ampere (MKSA) system proposed in 1950. The implementation of SI when reporting clinical laboratory results has long been a concern of the International Union of Pure and Applied Chemistry (IUPAC) and the International Federation of Clinical Chemistry (IFCC) which recommended in 1966³ the litre as denominator in concentration units involving volume, amount of substance (unit mole) whenever possible, and preferential use of whole multiples of three as powers of ten. These recommendations do not advocate full implementation of SI but recommend restrictions by limiting the clinical laboratory to 'favoured' SI units.

Any measurement in science or medicine can be adequately and unambiguously expressed with SI units, and, with few exceptions, no basic change in units used in the clinical laboratory is mandated. SI does not stipulate that

concentration measurements must use the dm³ (litre) as unit of volume, nor does SI stipulate that analyte concentration must be reported as substance concentration, that is mole/dm³(litre).

The articles "Americans retreat on SI units"¹ and "NEJM restricts use of SI units"² thus do not reflect accuracy in reporting, and contrasting "conventional units" with "SI units" is misleading. Within SI, expressing cholesterol concentration in mg/dL is just as valid as expressing results in mmol/L. The only 'conventional' units in general use that are non-SI are the mm of mercury and the (international) unit for enzyme analyses.

More appropriate headings in the *British Medical Journal* and in *Nature* would thus have been: "Americans (NEJM) lift restrictions on the use of SI units".

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1. Nylenna, M., & Smith, R. *Br. med. J.* 305, 268, (1992).

2. Watson, T. *Nature* 359, 175 (1992).

3. Dybkaer, R. & Jorgensen, K. *Quantities and Units in Clinical Chemistry including Recommendations 1966* (Williams and Wilkins, Baltimore, 1967).

Beyond belief

SIR — Stuart Sutherland (*Nature* 361, 292; 1993) berates John Polkinghorne for berating Lewis Wolpert who berates the whole sorry business of science-and-religion, and I wish to God it would stop. What is the point to all this earnest repartee, this desperately serious self-addressing, this Woolgathering in the Outer Homilies?

Most normal people cease such debate by the age of 14, and even renowned British scientists, one cannot help hoping, should leave off at around 50. Renowned British scientists who insist on believing that the Universe was created and is being maintained and guided by supernatural forces and/or beings whose nature lies forever beyond our comprehension are not going to be swayed by rational discourse, logical analysis, empirical evidence and sanity. If they were subject to these influences they would not believe the things they do in the first place and when they are also ordained priests they have embraced a style of now-you-see-it-now-you-don't in regard to Reason that is invincible to mere factual consideration, having long since soared beyond the gravitational pull of reality.

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Whale numbers in dispute

SIR — Dr Sidney J. Holt of the International Fund for Animal Welfare cites me¹ as sharing the view attributed to Professor A. S. Blix² that “any resource that can be harvested on a sustainable basis . . . should be harvested . . . Not doing so is a waste of resources”, and then goes on to ridicule this view. But, emphatically, I do not share this view, nor did I express any such view during the meeting in Stockholm on 7 December to which Holt refers.

That meeting was arranged by Greenpeace Sweden. Holt and I both have copies of the written verbatim report of the invited contributions. One of the subjects debated was “Is the Antarctic Whale Sanctuary Necessary?”, which referred to a proposal for a whale sanctuary in the Southern Hemisphere put forward by the government of France to the International Whaling Commission (IWC).

At the meeting, I argued against this particular proposal, but not against sanctuaries in general. On the contrary, I emphasized that “sanctuaries could be of value for research and as a management tool”. Last year, I expressed similar views to the IWC Scientific Committee, and Norway took a similar stand at the IWC’s annual meeting: “We are not in principle against sanctuaries, but in this case . . .”³.

Holt was present at both meetings. It is possible that his political interests are furthered by vilifying Norwegian scientists, for instance by deliberately misrepresenting my opinions, but that is hardly of benefit to the scientific debate.

Holt also misleads your readers by writing: “The present difficulty arises from the fact that the IWC Scientific Committee has agreed, unanimously, that these whales declined in number continuously from at least 1953 to 1983 (the last year for which there are appropriate data).” Holt is citing some words from the committee’s report⁴, but has added the word “continuously”, suggesting a steady decline towards serious depletion. But the data presented to the committee indicated that there was a “rapid reduction in stock size following the beginning of whaling”, and that the stock size was either constant or perhaps even increasing after about 1960. The committee eventually agreed “that there has been a statistically significant decline” between the early 1950s and the early 1980s, but did not express an agreed view about the size of the decline or the trends during that period.

Last year, the IWC Scientific Committee unanimously agreed to adopt 86,736 as the best estimate of the size of the Northeast Atlantic minke whale stock.

The 95 per cent confidence interval for this estimate ranges from 61,000 to 117,000⁵. The estimate is based on line transect methodology and sighting surveys and other field investigations carried out between 1987 and 1991.

Earlier estimates were based on mark-recapture observations of minke whales marked in the years 1974–78 and recaptured in 1975–82. Because the number of whales marked was small (333), the estimates were uncertain and were recognized as such at the IWC meetings from 1979 to 1984⁶. The estimates submitted to the Scientific Committee by Norwegian scientists for these years varied from 48,000 to 121,000, all with very wide confidence limits.

Vassili Papastavrou is thus mistaken in claiming⁷ that “[f]or several years, Norwegian scientists insisted on a figure of 113,000 obtained from marking experiments” and that “[t]here has never been a ‘previous estimate’ as low as 18,000”. (The figure I quoted to *Nature* was 19,000.) But in 1988, the IWC Scientific Committee discussed the results of the first sighting survey experiment in the North Atlantic (a pilot experiment in 1987) and agreed “a provisional estimate” of 19,112 (CV = 0.163) animals⁸. Although only a “provisional” estimate, this was much used by opponents of whaling as a good estimate of the total Northeast Atlantic minke whale stock in public debate even when better (and higher) estimates became available.

There is reason to believe that the current best estimate of 87,000 animals is biased downwards⁵. The seas off the west coast of Scotland (where they are being studied by Papastavrou), to the west of Ireland and in the Irish Sea, for example, are not included in the area of the 1989 sighting survey. Yet, as Papastavrou rightly remarks, these whales probably belong to the same genetic stock. Norwegian whalers formerly took substantial catches in these waters.

Papastavrou further claims that “estimates of depletion are more robust than estimates of stock number”. This is true only for a short time period; over longer periods, uncertainties in birth and death rates and their density dependence will strongly influence the estimates of depletion, as the IWC Scientific Committee acknowledged in its discussion in 1991⁴. Papastavrou then refers to “improved calculations presented to the IWC Scientific Committee in 1992” which in his opinion “strongly confirm that the catch limit should remain at zero”. These calculations were not presented to the committee as a full scientific paper and were “neither discussed nor reviewed by the

committee”⁵. Our own calculations, using the extremely cautious catch limit algorithm recommended by the Scientific Committee and accepted by the commission in 1992, result in quotas substantially larger than zero for the Northeast Atlantic minke whale stock.

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1. *Nature* **361**, 391 (1993).
2. *Nature* **360**, 523 (1992).
3. Verbatim Record, p.67, 1992 Annual Meeting, IWC.
4. *Rep. int. Whal. Commn* **42**, 68–69 (1992).
5. *Rep. int. Whal. Commn* **43** (in the press).
6. *Rep. int. Whal. Commn* **30** (1980) – **35** (1985).
7. *Nature* **361**, 391 (1993).
8. *Rep. int. Whal. Commn* **39**, 45 (1989).

Save the amber

SIR — Paul Whalley in his review of George Poinar’s *Life in Amber* (*Nature* **360**, 714; 1992) notes that DNA can now be extracted from insects encapsulated in amber, but does not say that extracting DNA from insects in amber requires that the specimen be pulverized. I suspect there will soon be a rush to crush even more amber insects into powder so their DNA can be sequenced.

This may not be a serious problem for common specimens, such as certain wasps and stingless bees in Dominican Republic amber. But what fate awaits rarer specimens? Will some of them also be pulverized so that their DNA can be checked?

DNA sequencing is a vital tool for investigating past life, but it is important that a system of peer review and approval be established to control the destruction of rare biological specimens, fossil and otherwise, for the purpose of DNA sequencing.

Meanwhile, scientists carrying out such work should carefully classify, document and photograph any specimen in amber before it is crushed. The venation of insect wings should be carefully recorded. It is especially important to document specialized external organs such as the spinnerettes of spiders.

Classifying amber inclusions can be difficult. Many have yet to be completely classified, so that researchers should perhaps concentrate on classifying what has been found in amber before destroying the specimens. They should also be aware of laws and regulations governing the destruction of fossils, especially those removed from museum collections.

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Rall's farewell to Frontiers

SIR — I shall retire as chairman of the Council of Scientists of the Human Frontiers Science Program (HFSP) in April 1993, and as *Nature* on several occasions has reported some dissension among the participants in this programme, I feel it relevant to record my observations.

The programme was devised and initially totally supported by the Japanese government but initiated only after lively discussion by a worldwide group of scientists who collectively established its goals. The concept developed was to support the most important areas of contemporary biomedical research on an international scale and with particular emphasis on interdisciplinary approaches. Support was to be provided for grants, fellowships and workshops which fell into the categories of "basic research for the elucidation of brain function" and "basic research for the elucidation of biological functions through molecular level approaches".

The programme began officially in the fall of 1989 and Sir James Gowans was inducted as secretary general in January 1990. The first grants and fellowships were awarded in April 1990. The review committees were constructed to have international representation and the grant requests were required to represent interdisciplinary and international efforts. In spite of an extremely tight deadline, Gowans was able to assemble competent committees and present the Council of Scientists with careful and critical reviews only a few months after the initiation of the programme. From the great interest manifested throughout the world it was apparent that this programme could fill a need for modern biomedical science, previously unmet.

So many excellent grant and fellowship applications were received in the first year of the programme that we were forced to reject, for lack of funds, many truly exceptional proposals. At that time, several of the national delegates of the Council of Scientists wondered if a somewhat more focused programme might more appropriately match the limited funds available. No action was taken or recommended. Subsequently the great success of the programme in continuing to attract superb proposals and the gradual increase in funding made the previous discussion appear to be somewhat premature.

Throughout all the discussions over specific grants or fellowships, I was unable to detect the slightest degree of national or chauvinistic tinge to the opinions offered. Obviously opinions differed, but more likely than not they reflected the field concerned; that is, neurobiologists contended that study of

neurobiologists contended that study of brain function was more important than anything else, and cellular molecular biologists vigorously defended their terrain. A view has been expressed that the HFSP should specify the nature of the interdisciplinarity it wishes to sponsor. All these questions are matters for the Council of Scientists and were discussed at its meeting last month. In general the council reaffirmed the original objectives of the broad interdisciplinary programme as it has developed.

Although the budget has increased significantly, non-Japanese contributions still account for less than a quarter of the total. The council and the newly designated secretary general, Dr Michel Cuenod, hope for and strongly support an increase in non-Japanese contributions to make the budget coincide more closely with the opportunities available. The unique characteristics of the HFSP deserve emphasis; it is completely international, both in a rigorous review process and in the grants themselves; it strongly supports a widely interdisciplinary attack on major unresolved problems; and the quality of the grant and fellowship applications is remarkably high. This programme, initiated by the Japanese and developed by an international group of scientists, is an unqualified success.

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Absolute truths

SIR — In the leading article "*Eppur si non muove*" (*Nature* 360,2; 1992) about the Vatican's recent rehabilitation of Galileo, on the one hand you conclude that "the Vatican owed him a less grudging recantation" and on the other hand you wonder whether the Earth goes about the Sun in any but a relative sense, adding that "prescient Galileo was probably too good a scientist to have committed himself to an absolute view".

This seems to miss the central point of the Galileo affair: it was precisely Galileo's insistence on an absolute view of the Earth's motion that got him into trouble. In the 1616 trial he explicitly rejected Cardinal Bellarmine's advice to adopt an instrumentalist view of the matter and to consider the Earth's alleged motion as merely a convenient hypothesis. Following in the footsteps of Mach, Duhem, Einstein and others, you imply that Galileo erred on this matter. If that is indeed the case then perhaps it would be more fitting for the scientific community to apologize to the Vatican for its past ridicule of the Roman Catho-

lic Church's assessment of Galileo's absolutism.

You express concern that "the Galileo business provides a perpetual licence for prejudice in the evaluation of discovery". In particular you cite the damage that can be done among the young by creationists. Yet you yourself note that scientific discoveries are not cut and dried. Indeed, as Popper, Kuhn, Lakatos and others have shown, both the construction and assessment of scientific theories are heavily dependent upon our prior religious and philosophical biases.

What the Roman Catholic Church and creationists have in common is the belief that, regarding the unseen world beyond our observations, divine revelation, in the form of the Bible, is more reliable than mere human speculation. Although such views may nowadays be very much in the minority, in the absence of clear, valid criteria for theory selection they can hardly be summarily dismissed. As history has repeatedly shown, a majority vote is not a reliable indicator for scientific truth. In short, you should be more charitable to those whose religious prejudices differ from yours.

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Music of the stars

SIR — You report the use of pulsars in musical performances (*Nature* 360 96; 1992). The first musical performance involving a radio pulsar as a 'live' performer took place in Western Australia in 1988. The occasion was the Fifth Marcel Grossman Conference on General Relativity and Gravitation. Perth's Tunc Ensemble, which included saxophone, flute and didgeridoo, played to the live sounds of Pulsar 1749-28. The pulsar signal was received by the Molonglo Radio Telescope in New South Wales. The signal was beamed across the continent by microwave link, and beamed directly into the auditorium of the Perth Art Gallery. The wave form was displayed by laser beam.

The particular pulsar was chosen for its drumlike period, its very strong signal and its occasional pulse nulling which gives an interesting syncopated rhythm. The audience included John Wheeler, Murray Gell-Mann and Sir Ninian Stephen (Governor General of Australia). The entire performance was recorded on video and is available from the University of Western Australia.

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After the Gold Rush

California, at the forefront in science and technology, now has to cope with the consequences of its success in lean times.

A RUDE saying about California holds that it is like a bowl of cereal: populated by nuts, fruits and flakes. Only in San Francisco, for example, would a city's planners impose a grid-square of streets without regard for the rugged underlying topography. The result is a road network that requires the nerve of a Finnish rally driver to negotiate. But had the planners built around the hills, San Francisco would lack its unique character, imposed by a people who will never take 'no' for an answer, however quixotic the task.

California was an outpost of the Spanish empire for 280 years, longer than the history of the United States. It was claimed for Spain in 1542, 78 years before the Mayflower set sail for the eastern seaboard: when the Declaration of Independence was signed, the State had been Spanish for more than two centuries. But California was too distant for a fading imperial power to maintain, and became a province of Mexico. Even then, this vast region was so remote from any centre of government that it was effectively open to any who would stake their claim. In 1846, the population of what might loosely be termed Americans rebelled against Mexico. California became a Territory in 1848, and statehood followed soon after, together with the Gold Rush. The veneer of civilization that eventually caught up has done little to damp a pioneer spirit that would drive a family 2,000 miles across an empty continent on the rumour of riches.

The freewheeling spirit of this cosmopolitan population was ideal ground in which education and inquiry could take root. Hardly more than twenty years after the Gold Rush, what is now the University of California at Berkeley opened its doors. The rapid arrival of a population free from the restraints of tradition and established values engendered a kind of lateral thought, in which even the most outlandish ideas were considered seriously. Marinated in such ideal conditions, it is perhaps less surprising than it might seem that Californian science has grown from nothing to the best in the world, in slightly more than a sin-

gle human lifetime. Thanks in large measure to its science, California has the eighth largest economy in the world. The sunshine adds its blessing in the form of an elysian climate and a cornucopia of produce, but California also houses the now-huge semiconductor industry, while new biotechnology companies continue to set up shop.

But the very things that made California rich are now making it poor. The intellectual climate, so welcoming of the unconventional, has worsened recently with the removal from office of state schools' superintendent Bill Honig, who had allegedly authorized \$337,509 for the

tory, California's economy has taken a nosedive. The end of the Cold War has brought its vital aerospace industry crashing down amid the deepest global recession since the 1930s.

But the biggest problem is not dearth, but superabundance: too much of California's most important resource — immigrants. Colonists still pour into the State on the promise of a more abundant life, if not actually gold in the mill-races. The Mexican flag has long since gone, but Spanish is the lingua franca in many Californian schoolyards. Until now, rapid economic growth has enabled the State to lavish money on enlightened educa-

tional and welfare policies, such is its commitment to the immigrants that have, historically, put the gold into the Golden State. Ever since its piebald beginnings (and perhaps because of them) California has been far more tolerant of racial and social diversity than elsewhere — the colonization of San Francisco by the gay community, and the extraordinary ethnic mix on Californian campuses are just two examples. But California can support its immigrants no longer.

The recession, exacerbated by the decline of aerospace, has forced unemployment up to 10 per cent. The growth areas are in biotechnology and other kinds of high technology, which are hardly labour-intensive: they will not make a significant difference to jobs for another twenty years. In the meantime, Californians must turn to their by-now-traditional trump card to get out of the

mire — lateral thought. Amid the gloom are signs of a recovery, fuelled by the adventurers who continue to regard adversity as an opportunity.

But still the immigrants pour in, like the enchanted broomsticks that plagued the Sorcerer's Apprentice (memorably realized in Disney's *Fantasia* by that famous Californian, Mickey Mouse). For California, as for poor hapless Mickey, the only way out may be by the grace of a magic wand. The Gold Rush is finally over. What California needs now is fairy dust. On past form, it may get it. □



Quality Education project, a nonprofit organization run by his wife. On January 29, Honig was branded a felon, ordered to refund that sum (plus a \$10,800 fine) and serve four years' probation. Honig is an outspoken supporter of evolution, and many fear that the severity of his punishment is a sign of California's energetic creationists at work. If so, the danger is that the state's schoolbooks will suffer the debilitation of creeping creationism. And where California leads, others will surely follow.

Second, and for the first time in its his

This supplement was written by Henry Gee, with assistance from Gregory Gasic. *Nature* would particularly like to thank those who generously gave up their time in aid of this project, and apologizes to those whose work, for reasons of space, has not been described in detail.

Unlawful entry

MORE than one in five Californians were born in other countries: many of the rest were born to foreign-born parents. Since its foundation, California has derived much of its economic (and scientific) strength from the vigour of its foreign-born residents. Thanks to its immigrants, this one state is proud to have the eighth largest economy on the world, with an aggregate personal income of around \$650 billion. But one can have too much of a good thing. Immigration, especially illegal immigration, is one of the three principal causes of California's current economic catastrophe: the others are the deep global recession and the downturn in defence spending. The first will solve itself, eventually; the second, which has particularly affected southern California, will shift after a certain amount of readjustment (see below). But even after the current recession ends, immigration will force State expenditure to outstrip income well into the next century.

The first proposal for the budget for the next fiscal year, 1993-94, is that it will be balanced, at great sacrifice. There will be no tax increases, and across-the-board cuts any deeper than those already instituted will be detrimental: therefore, the budget reflects some hard choices. For example, job-creation schemes and K-12 (kindergarten to 12th grade) education will assume top priority, but support for higher education will be cut by about 10 per cent. Ultimately, recovery depends on long-term economic restructuring rather than simply raising taxes and cutting spending, and there is no shortage of initiatives, some state-sponsored, to stimulate the economy.

Crucially, the budget is predicated on immediate injection of federal funds. The state's only solution is to hold its own citizens hostage to its demand that the federal government meets its obligations, and compensate California for its share of the federal immigration burden. If these funds are not forthcoming, further cuts will have to be made.

In the good old days, when California grew rich making fighter planes for Uncle Sam, the state received more in federal expenditure than it gave back in taxes. From a peak in 1982-83 of \$1.22 federal input for every \$1 going out as federal taxes, the tables have now been turned so that by 1988, the state was receiving just 91 cents for every dollar paid back in federal taxes. What with the federal budget deficit and the recession in general, this deficit is likely to widen still further. Indeed, it will be ripped wide open should the new administration cut defence spending further and target rich people for more taxes. In 1990, 12 per cent of all federal income tax returns came from California: but 16.5 per cent

of those returns declaring gross incomes of \$100,000 and above were filed from the Golden State. The governor has been labouring to close the gap, by lobbying for a large share of the \$1.5 billion federal defence conversion programme, but for the time being California will send more dollars to Washington than it gets in return.

Immigration will make the problem far, far worse. The United States as a whole has 20 million foreign-born residents, one-third of whom arrived between 1985 and 1990, and a quarter of whom live in California. The state will be obliged to provide healthcare and education to all these people as of right, while at the same time sustaining a relative decrease in the number of taxpayers.

Unfortunately for California, immigration policy is dictated from Washington, not Sacramento. The leaky borders are a federal responsibility, but federal law requires states to provide a variety of healthcare, child benefit and superannuation benefits to immigrants, legal or otherwise, without prospect of reimbursement from Washington. For example, \$3.6 billion of California's budget this year is being spent on K-12 education for illegal immigrants, or the citizen children of immigrants generally.

To make matters worse, the Federal Immigration Reform and Control Act

(IRCA) of 1986 granted amnesty to illegal immigrants residing in the US since 1982. Under IRCA, Congress authorized \$4 billion of grants to help states assimilate their immigrants, but California received \$467 million less than the 60 per cent share to which it felt entitled. IRCA also required the US Attorney General to reimburse states for the cost of imprisoning illegal immigrant criminals. 12 per cent of Californian prisoners fall into this category, costing \$243 million annually, and Congress has appropriated not one cent.

In this way, California is paying heavily for an immigration policy imposed from above, something that it can no longer afford. In a bold political gamble, the governor has demanded — as part of the budget proposals — that Congress appropriates \$1.5 billion, no later than 15 May this year, to compensate California for the cost of assimilating its immigrants. Otherwise the budget will be in deficit before the 1993-94 fiscal year even begins.

If these funds are not received, the state will eliminate a variety of sensitive health and welfare programmes worth \$1.2 billion, and carve further huge chunks from its other programmes, confident that the federal government, not Sacramento, can be blamed. Higher education, subsidized to the tune of \$4.7 billion, is a tempting target. Give us the money, Bill, or we shoot this professor. □

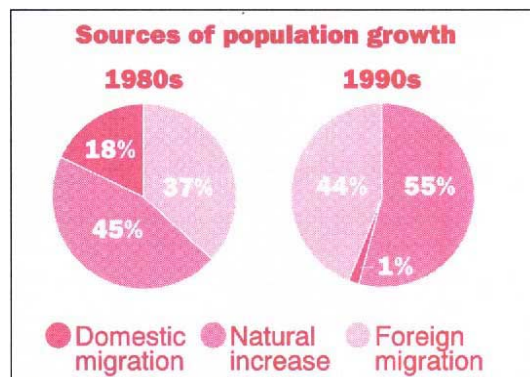
Women and children first

At present there are approximately 250 million Americans, and 31.3 million (about 13 per cent, or one in eight) are Californians. Every year welcomes another 600,000, and in the year 2000 there will be 36.4 million Californians, an increase of 6.4 million in the 1990s (6.2 million in 1980s). But 2.5 million (39%) of these people will be under 17 years old: the increase in this sector was 1.5 million in the 1980s. The taxpaying population (18-64 years), though, will grow more slowly in the 1990s than in the 1980s; 3.5 million compared with 4 million.

Natural increase (the number of births over deaths) will account for 55 per cent of this growth in the 1990s compared with 45 per cent in the 1980s: a decline in individual fecundity will be offset by a larger population of women of childbearing age (15-44 years); 8 million in 2000 compared with 7 million in 1990.

Although natural increase will reduce the overall contribution of immigration from 55 per cent to 45 per cent of population growth in the 1990s, the percentage of immigrants from abroad will

increase in spectacular fashion. In the 1980s, 18 per cent of the population growth was as a result of Americans moving to California from other states,



37 per cent from foreign immigration. In the 1990s, almost all immigration will be foreign: 44 per cent of the growth, compared with 1 per cent from domestic immigration. This sharp decline in domestic immigration may be related to the recession. In the year 2000, a far higher proportion of Californians than today will be the children of immigrants from abroad, who will constitute a formidable drain on the state's resources. □

Can they do it again?

THE budget for the present financial year 1992–93 assumed a modest economic recovery this spring. This has clearly not happened, and the financial year 3 will end with the State in debt by \$2.1 billion, or perhaps slightly more. This does not seem too bad in context of the revenues received, except that revenues for the next financial year are expected to decline sharply: the first time this has happened since the Depression of the 1930s. The proposed budget for the General Fund (excluding Special Funds for specific purposes) for 1993–94 is \$39.9 billion, a reduction of \$1.07 billion on the previous year, \$37.8 billion of which will be available after paying off the debt. 40.2 per cent, or \$15.2 billion of this is tied up in mandatory obligations such as education, leaving \$22.5 billion for health, welfare, public safety, higher education and all other expenses: \$151 million less than last year. \$31 million will be set aside as an emergency fund.

Against this decline in revenues, expenditure commitments are charitably described as unrestrained. For example, the Medi-Cal (medical welfare) caseload will increase by 7.5 per cent next year, some three times the general population increase. All in all, available revenues will meet only 80 per cent the expenditure demand. Deficit spending is out of the question for a variety of reasons, hence the severe cutbacks in a variety of programs necessary to balance the budget.

The state has a number of additional crosses to bear. Perhaps the most notorious is Proposition 13, a constitutional amendment sponsored by private citizens and passed 2:1 by the voters in 1978, whereby the tax of 1 per cent on property value is fixed, and reviewed only when the property is sold. The idea was to provide tax relief for residents burdened by rising local taxation in a booming property market. This has since had three effects. First, the state had not been able to take fiscal advantage of the extraordinary rise in property values in recent years. Second, this very rise is a disincentive to property transactions, which will be subject to sudden, large tax re-assessments. Third, people in the same neighbourhood pay wildly different sums for the same services, depending on the history of their individual properties.

The immediate effect of Proposition 13 was to cut local tax revenue by two-thirds: the state, which had passed the amendment, had no option but to bail out local governments and assume control of a wide range of local services such as schools, emergency services, jails, trial courts, land registration, and planning. The state can no longer afford this subsidy, and the upcoming budget proposes

to eliminate a great deal: school funding will be returned to the mix of State and local sources that applied before 1978.

The long-term outlook is gloomy to the point of opacity. Largely because of immigration, mandatory expenses will grow faster than the ability of the state to meet them. Throughout the 1990s, K-12 enrollments are projected to grow by 37 per cent (23 per cent in the 1980s); numbers of people eligible for child benefit (Aid to Families with Dependent Children, or AFDC) will grow by a staggering 61 per cent (14 per cent in the 1980s),

AEROSPACE

Crash and burn

OF the 800,000 jobs lost in California over the past two years, a quarter were directly or indirectly related to defence and the aerospace industry. In southern California alone, 100,000 aerospace engineers may be out of work.

Twenty-two cents of every dollar spent on defence in the United States are spent in California, almost double per capita that in any other state. So it is no surprise that when the Cold War thawed, California got chilblains. Under the Bush administration, the federal defence budget was expected to fall by 30 per cent between 1991 and 1996. If President Bill Clinton's pre-election statements are anything to go by, the figure could be as high as 50 per cent. That this affliction struck on the brink of a recession was hard luck.

To make matters worse, California's high taxes, cost of homes, forward-looking environmental protection regulations and expensive workforce compensation arrangements have driven much of what is left of the aerospace industry.

In previous recessions, aerospace companies have survived through diversification. This time they are consolidating their core areas, breaking down peripheral activities and selling off the parts. For example, General Dynamics has sold its missiles unit in San Diego to the Hughes Corporation, and its fighter aircraft division in Fort Worth, Texas, to Lockheed. There is a perception that the weaker companies will fall prey to the strong, and that the number of key players in US aerospace will have halved by 1996.

This trend does not mean that diversification is no longer an issue in the present recession, heavy investment in plant and equipment means that there are some operations that simply cannot relocate, and diversification is the only option.

This has meant two things for aerospace companies. First, a broadening of

and recipients of state healthcare (Medi-Cal) will almost double — a barely credible 98 per cent (27 per cent in the 1980s). The tax base, however, continues to be eroded by unemployment (projected to improve from 10 per cent to 9.5 per cent by 1994), the relative decrease in the number of taxpayers, and the migration of middle-aged high-earners out-of-state.

As a consequence, growth in expenditure will continue to outstrip revenue throughout the 1990s, long after the present recession is history. For example, revenues in the financial year 2001–02 will be an estimated \$68.5 billion, and expenditures \$83.4 billion. □

core activities into the civil sector. This broadening has spread to encompass some distinctly un-traditional partners. With Rockwell, Lockheed is working with Russia to develop a lifeboat for the Space Station out of the present Soyuz spacecraft design. This, in turn, has led to a 50:50 venture between Lockheed and Russia to market the massive but reliable Proton booster internationally.

Second, aerospace managers have applied some lateral thinking. If the design of a stealth bomber or a space shuttle requires some facility with large, complicated systems, then the same skills can be applied to civilian projects: among many other activities, Lockheed has built and runs an airport in Toronto, and with Rockwell and others has been working on metropolitan transit systems. Closer to home, it manages the system that collects fees in San José's parking lots. The division of Lockheed that coordinates these activities is the fastest growing part of the company, and around 40 per cent of Lockheed's research concerns software design and analysis — for both military and civilian use.

Adaptation is more than a problem of re-tooling, it requires a change in culture. Most of all, aerospace companies are not used to the cut-and-thrust of the marketplace, which runs counter to everything they have learned.

This cultural change is happening — it must happen — and there is no sense that the aerospace industry in California is headed for extinction, although an overall sense of the sort of things it will do in future is unclear. Perhaps they will do a bit of everything. In that the mighty Californian aerospace industry of today was built by the flood of otherwise unoccupied engineering graduates in the 1960s, the past offers a lesson. Today's unemployed engineers will have to find something to do: now, as then, it is in the interests of the state that they do it in California. □

Insurmountable opportunities

THE University of California (UC) has a problem. Budgets are shrinking at a time when student demand has never been higher. And while the UC is obliged by statute to educate California's best and brightest, it is running out of places to put them.

At the root of the problem is the Master Plan for higher education in California, whereby UC can select its students from the top eighth of the state's high-school graduates, the state universities (which do not award research degrees) from the top third, and the two-year community colleges from everyone. The plan was instituted in 1960 and its success in producing an educated workforce may in part account for California's economic vitality since the Second World War, not to mention the extraordinary growth in the UC system in the 1960s.

The problem now is that with a population growth of more than 620,000 a year, there are simply too many students. In theory, the state's two public university systems (UC and California State University, CSU) can be selective about their admissions: in practice, a university education is now seen as an entitlement by those who qualify, a view that UC administrators are proud to service and which would be politically hard to counter.

In the late 1980s, UC laid plans for a tenth campus to meet the demand. It looked at three possible sites in the Fresno area of the San Joaquin Valley, where people are beginning to outnumber orange trees. The plan was to open in 2000, but the cuts have put this back to 2005 at the earliest. At present, UC has 166,000 students (12,000 more than it has funds for) with 5,000 fewer staff and \$700 million fewer dollars than it needs. Students at the existing campuses will just have to cuddle up, and pay more for the privilege: 1994's seniors will leave UC paying double the tuition fees they paid as freshmen in 1991. In five years, there may well be no more room at the inn, no matter how high the fees.

Of course, UC is in the same boat as any 150,000-strong corporation in California — except that it does not have the option of moving out of state. Faculty may have to accept a five per cent cut in salaries next year, after two years of essentially no pay increases. In another money-saving effort, 4,000 senior staff and faculty have accepted tempting early retirement packages over the past two years, and another 1,000 posts are to disappear, mostly by natural wastage. The concern is that with retirement-age faculty already pensioned off, any more voluntary redundancies will attract talented faculty in their 50s, who will go on to accept standing offers from institutions

able to offer better salaries and conditions. The result will be a diminution of UC's enviably high standards — and ultimately of the economy of the state, so close are the ties between town and gown. There are already signs that the university is less persuasive in recruiting its first choices than it was.

It is at times like this that people start to ask whether the university really needs an expensive central administration: the president's office on the twenty-second floor of an office block in downtown Oakland seems a long way from the tie-dye stalls along Berkeley's Telegraph Avenue or UCLA café society.

The answer has to be 'yes', at least in principle. Kumar Patel, fresh from Bell Laboratories and brand-new vice chancellor for research at UCLA, sees the recession as an ideal time to make decisions about concentrating subject areas in particular campuses and avoiding duplication of effort. Such strategic planning is best done from the twenty-second floor than from the ground.

Without the president's office, the state would find it very hard to get an all-round idea of what is going on at the nine huge campuses, five busy teaching hospitals and three national laboratories that comprise the University of California. In a sense, the UC administration is like the Universities Funding Council in the United Kingdom; it is an interpretative tier between government above and the swarm of academic institutions below.

And there is one piece of good news—

the state is sympathetic to the university's needs, even if it cannot do much about it right now. The present "fiscal peril" is "not a function of the governor or the legislature not liking us, or penalizing us", says UC vice president Bill Baker, who as the man in charge of the budget has more money at his disposal than do most of America's 'Fortune 500' corporations. "They'd give us what we asked, if they had it, I'm convinced of that." This was not always the case. When Ronald Reagan and Jerry Brown were state governors, education was less favoured. But discretionary funds for higher education must take their place in a budget over-committed to mandatory expenses.

The University of California receives about 25 per cent of its funds from the state. This core budget was \$2.135 billion in financial year 1990–91, but has now shrunk to \$1.881 billion — a reduction of \$254 million in three years. The university requested \$1.99 billion for the next financial year, but will not get it: the next budget proposes a further cut, to \$1.743 billion, a cumulative loss of \$392 million, or 18 per cent, on the 1990–91 figure. Baker says this is "the worst fiscal problem we've had, maybe ever". He says that it may be even worse for the university than the Great Depression.

The core budget pays the salaries and keeps the buildings from falling down: it cannot be augmented by the remaining 75 per cent of UC funds, which are in the form of grants and receipts for particular purposes. The university receives 11 per cent of all federal contracts and grants, but this is of little help to the core bud-

The Cal factor

THE UC has grown into arguably the world's finest public university in little more than 100 years. Why? California was a magnet for academics from the early 1920s for a variety of reasons. Imaginative recruitment methods compensated for sparsity of population in the Depression — when people were attracted by low salaries when the alternative was none at all. By the 1930s UC had an enviable cadre of physicists, among them the core of future nuclear weapons projects, which explains why it is now the custodian of no fewer than three National Laboratories: two within the state itself, the third (Los Alamos) in New Mexico. After the Second World War, California's economic miracle — founded in large measure on defence and aerospace and a rapid influx of technically gifted immigrants — bathed the UC in money. At the same time, the State accorded the UC a large measure of the political freedom

to use that money to its own ends: rather than being administered from the Capitol (as is the CSU system and most other State University systems elsewhere), the UC is run by its own Board of Regents. The 'X' factor is access to quality. Whereas California's private universities, such as Stanford and Caltech, are extremely good, they are very small: this has enabled the UC to attract a high proportion of the best faculty and students. This has not worked out elsewhere: the State of New York, for example, has arguably failed to produce a public university system that matches UC in quality and quantity, largely because of competition from large, private schools such as Columbia and Cornell. The quality of UC education is high because it starts with the best ingredients. "If you want to make a silk purse out of a sow's ear", says UC senior vice president Ronald Brady, "start with a silk sow." □

get. Nevertheless, the state recognizes that it gets a good deal from the university: "for every state dollar that we get, we bring in about three times that", says Baker.

As for a solution, there is no shortage of ideas — but no simple answers, of course. A state tax for higher education is being considered, although this would be "politically full of sharks" concedes Baker. More seriously, Jack Peltason, president of the University of California, has announced a series of 'strategic initiatives', one of which is a formalization of the way UC keeps track of its patent royalties (see below, and *Nature* 360, 701; 1992). Another is the California Business-Higher Education Forum, an organization of up to 60 business and education leaders. It decided at its first meeting in February to study how the Master Plan can remain viable with current constraints.

Even the idea that the university could occupy one of the two national laboratories within the state that it runs under contract from the Department of Energy has been considered (the third is at Los Alamos, in New Mexico). Faculty and buildings are already in place, and the need to house students is now more pressing than the need to make atomic

Technology transfer

GENENTECH and Chiron face each other across San Francisco Bay. Whether measured by research output or citation impact, they are clearly in the biotechnology superleague. Their combined citations are more than half again as many as those of the other eight top ten US biotechnology companies put together: and they have made a truckload of money. Yet their success stems from the research that their founders, respectively Herb Boyer and Bill Rutter, did at the University of California. "Those are models that people always point to and say 'boy, you need to do more of those'", says Ronald Brady, senior vice president of the University of California (UC), who seeks to corral more of the money generated by UC research back into UC's now threadbare pockets.

But Brady is not just a patent policeman. By fostering stronger links between academic institutions and business, UC could use its royalties as seed-money that could by 2001, inject \$9.5 billion into California's economy — a sum greater than five times UC's total annual budget.

As part of UC president Jack Peltason's latest round of initiatives to fix UC's health, the university will set up two corporations to foster technology transfer, in what has been called the Enhanced Technology Transfer Program. One of these, the nonprofit UC Technology Development Foundation (UCTDF) will retain UC patents as an agent of the university's Board of Regents, and offer inventors legal, technical and financial services.

The other, the for-profit UC Technology Development Company (UCTDC), will evaluate an invention or discovery that may be close enough to the marketplace to be licensable and, if it is, nurture it with seed-money as a start-up company until it can be sold on to private sponsors: licence agreements from such a start-up would be managed by UCTDF, or UCTDC may wish to retain an equity.

Both companies would be financed by UC's royalty income, which stood at \$28 million in fiscal year 1992–93. By 2000, they plan to foster more than 20 start-up companies, and to award "gap funding" to help 100 inventions a year to the marketplace. The enhanced royalty income generated by these activities is expected to exceed \$222 million by 2001. But one thing leads to another, and jobs created by the ventures could result in an accumulated bonus to the state economy of \$9.5 billion by that date.

At present, the state receives 25 per

cent from UC's patent income, but in the budget proposals for fiscal year 1992–93, Governor Pete Wilson welcomes Peltason's request to waive this tax, in favour of additional funds for the technology transfer programme.

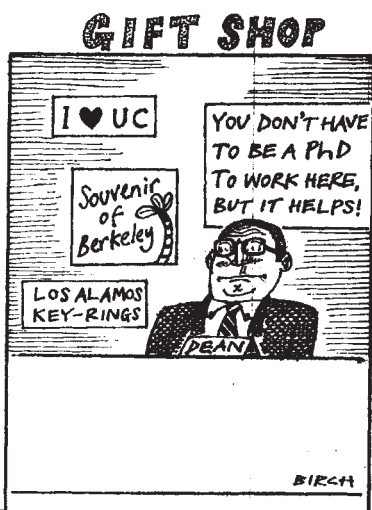
It is heady stuff, but built on the underlying philosophy that the entire stance of university research must adapt to straitened circumstances or perish. "Everybody agrees that we have put together a very powerful educational apparatus in this country. But we have not done well in terms of transferring that knowledge to the wealth-making part of society", says Kumar Patel, the new vice chancellor for research at UCLA. "The linear idea of transferring technology from researcher, to engineering, to manufacturing, to marketplace somehow just does not work. It takes too long, and if you cannot increase the velocity of what I call 'technology insertion', then we are dead."

The linear regime also creates an intellectual pecking-order that can be detrimental to economic health, in which research is regarded as more 'honourable' than engineering, engineering and more honourable than marketing, with salesmen at the bottom. But research that produces more people with PhDs will have little immediate impact on the economy.

The answer is to discuss ideas with industry while they are still at the bench-top stage. This is clearly an aim of the enhanced technology transfer initiative. Brady says: "The business community feels very strongly that there needs to be an increased interaction between the university community in its research capacity and their activities, directly".

The whole package is more than a couple of new initiatives. What it amounts to is a gross phase-change in the way academics see themselves and their activities. "A substantial number of faculty members are genuinely scared", says Patel: "then there is a small group of faculty members who are genuinely excited about the change, because they see that this is the vindication of what they had believed for many, many years, namely that university research must also be relevant research."

If even one of the 20 or so companies that UC hopes to engender by the year 2000 is another Genentech or Chiron, then everyone can go home and open the champagne. But Boyer and Rutter managed quite well without UC's initiatives. May not technology transfer be better managed at the bar-room-chat level, than from an office 22 floors above Oakland? Time will tell, but UC finances are in poor shape for a long haul.



bombs. Could the tenth campus be UC Livermore, rather than UC Fresno?

The university is nevertheless convinced that, in the long run, it will solve its problems. Its worries concern the immediate future. "In ten years time we'll be jammed to the rafters with students", says Baker. But in 20 years, he thinks, the problem may well have solved itself: more remote education by computer could be a feasible option by then. Kumar Patel is also confident that the university will rise to the challenge, just as Californians have always done. His favourite philosopher is a cartoon character called Pogo, who reminds us that "we are surrounded by insurmountable opportunities". □

I want a new drug

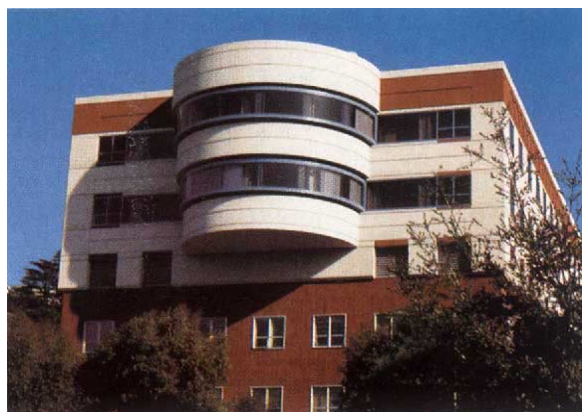
IN July 1981, the San Francisco General Hospital saw its first case of Kaposi's sarcoma. Twelve years and thousands of deaths later, nobody would argue that the best and most informed care of AIDS patients comes from work at SF General.

Similarly, it would be hard to deny that the epidemic has had a profound effect on society outside the hospital. The impact of AIDS has been so great that nobody has escaped — in a city where even daytime radio programming has an explicitly homosexual cast, you cannot help becoming involved. Today, about 60 per cent of homosexual men in San Francisco are infected, probably the highest density of infection anywhere in the developed world. "It colours everything we do out here", says activist Martin Delaney, "the gay community, to a large extent, is about addressing AIDS. It has to be, because it's literally a war: your entire community is under siege."

Over the past decade, the traditional lines between researcher, physician, patient and activist have all but washed away, leaving a loose network of people of less-well-defined activity, but all concerned in fighting AIDS. Brook Byers, venture capitalist, lives in a well-heeled, tree-lined district overlooking the Golden Gate. He kick-starts companies such as Genentech, long involved in AIDS research. Linda Sonntag, chief executive officer of another company, SyS-temix, lives around the corner. SyS-temix researcher Mike McCune (who lives in the same street as Sonntag) is developing a strain of SCID mouse with a transplanted human immune system, research that suggests radical therapies against AIDS. McCune is a member of the Immune Restoration Think-Tank, an independent study group organized by

Delaney's pressure-group Project Inform (PI), one of the longest-established AIDS awareness groups. Project Inform has an office in the Castro on the other side of town, the epicentre of San Francisco gay life.

Delaney achieved some notoriety in the mid-1980s for the organization of alternative trials of the experimental



The new Gladstone Institute of Virology and Immunology has a particular focus on HIV and AIDS.

drug GLQ223, a substance extracted from Chinese herbal remedies and popularly known as Compound Q, in an attempt to subvert the usual testing and approval procedure — which, to sick patients, seemed inordinately and perhaps lethally protracted. (GLQ223 is currently in Phase II efficacy trials at SF General.)

One of the doctors who participated in the alternative Q trial, and also a member of the think-tank, is general practitioner Larry Waites, who lives in a leafy street of terrifying gradient not far from the PI office. As one of five doctors in the 450 Sutter medical practice, he has

1700 HIV-positive patients on his books. He and others have been able to work with drug companies, the Food and Drug Administration and the hospitals in a programme of community-based clinical trials. And back at SF General is Warner Greene, keen to roll up his sleeves as director of the brand new Gladstone Institute of Virology and Immunology at SF General and get on with some fundamental research, but mindful of the activist contribution to AIDS care.

All in all, AIDS has produced a society with a distinctive character found perhaps nowhere else on Earth. It is as much a feature of the city as the cable-cars, the Golden Gate and the vertically challenging streets. In this context, it is perhaps interesting that the single most generous private donation to the AIDS programme at SF General came not from an endowment or foundation but from a local rock group called Huey Lewis and the News. In 1983, long before the Q controversy, the

group had a hit song called "I Want A New Drug".

When AIDS struck, it struck hard. In a single year, 1982, 21 per cent of the uninfected gay male population became infected, and for some reason not yet known, many of those infected early, died early. "Soon everyone, and I mean everyone, had a friend who was dying", says Delaney. "It seemed as if the entire city in the late eighties was in mass depression", remembers Waites. "It [AIDS] has already completely changed our lives", says Warner Greene: "there is almost a paranoia about the virus. I don't know if we can equate it to the bubonic plague of the Middle Ages, but certainly it is the most serious epidemic that we have faced in the last 100–200 years."

Ultimately, HIV's biggest mistake may have been that of all the places in the West to unleash its initial onslaught, it chose a city in California, where people are disinclined to accept that impossible tasks must always remain so. "The pioneer spirit is still very much alive in California", says Waites, "and people don't accept the status quo the way they would on the East Coast, where they would say 'Oh well, there people are going to die, let's just make them as comfortable as possible'. In the Immune Restoration Think-Tank, we don't accept the fact that people with less than 50 T cells [per ml] are automatically going to die. There must be something we can do to save these people, and by doing this, what we learn will help us to treat people with less damaged immune systems." □



Sf General takes to the streets.

Clone your own legend

It is raining, but the last two bronzed customers at the bayside plaza café aren't leaving. This is Genentech, so they are obviously scientists in animated discussion. Hold on, these guys aren't going anywhere. They are statues of Bob Swanson and Herb Boyer, at the moment when, over That Beer, they founded Genentech, not even 20 years ago. And

years in Kansas; "and there is a faith that — even though it's not exactly clear what's going to come out of it — then it may well turn into something that's of practical value as well. That's something that the founders believed early on, the idea that you do good science and everything'll come out OK in the end."

Because the company devotes half its \$500 million annual income (all of it from product sales) to research and development, these projects can get off the ground at tremendous speed with the minimum of bureaucracy. Larry Lasky came here from Caltech 11 years ago. Sometime later, at Genentech, he was working on a project to develop soluble CD4 as a candidate AIDS vaccine. It did not work, so he decided instead to try out the burgeoning field of selectins, with half an eye

high spirits (most people here are thirty-something.) "Genentech has always been a place where humour is very important", Lasky says, "practical jokes, pie-fights, and immature activities have always been a part of this place. I hope they always will be. It's playfulness." Phil Berman is one of the older members of staff, but declines to reveal just how old. "I'm very immature for my age", he admits. □

BIOTECHNOLOGY

Adventure capital

BEHIND every Herb Boyer or Bill Rutter, of course, there is a venture capitalist like Brook Byers of Kleiner Perkins Caufield and Byers, a firm highly rated for its skill in nurturing start-up companies. Venture capitalists are entrepreneurs who do technology transfer: from a scientist with a big idea, they assemble the management team, hire the scientists, put together physical plant, and most of all, get hold of the money. Clearly not everyone is cut out for this kind of work — not

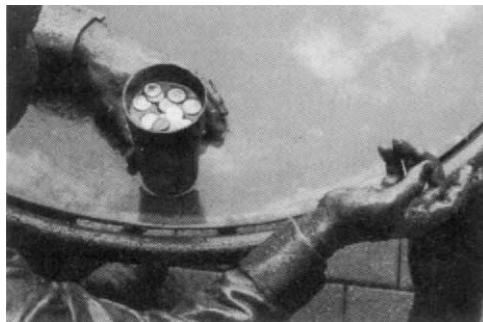


Permanent fixtures — Bob Swanson and Herb Boyer.

as if to votive ikons, their beer mugs are full of money. To a European, it seems hardly seemly that such a memorial could even be considered this side of 1776, let alone 1976, never mind that both protagonists are still alive. Such is the local mythopoeia in compensation for lack of history.

The Bob'n'Herb statue stands amid a brand new complex of buildings overlooking the bay, intended to house Genentech's entire research staff of 400. The change is long overdue. "For the last ten years we worked in horrible conditions in a building that was a warehouse, that had no windows, a very poor ventilation system and a roof that leaked every time it rained: but people didn't care because the work was exciting", says Phil Berman (once a postdoc at the Salk: "the quintessential California dream lab — by the beach"), currently working on antiviral vaccine strategies. Genentech people tend to be stayers, starting at the bottom and working their way upwards. This, of course, imbues them with a strong sense of identity and corporate pride.

Nevertheless, they are very much left to get on with the job — there is a hands-off management style that not even the arrival of Roche, with its 60 per cent stake in the company, seems to have changed. "There really is an attitude here that if there's something that is really novel and exciting from a purely scientific point of view, then it's worth pursuing", says biochemical all-rounder Joffre Baker, director of Cardiovascular Research, who came west after eight

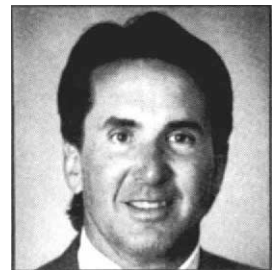


Money in the beer mugs.

on their possible role in inflammation. The project was off the ground within weeks. "If you get an idea at Genentech, within a couple of weeks you can put it into practice. The speed that you can do things is incredible", he says. And because of the on-site process engineering, the facility to make industrial quantities of protein is just across the street.

The mid-to-late 1980s were a time of rapid expansion for the company, and the problem became one of managing a workforce of over 2,000 without losing sight of scientific objectives. Genentech is now in the strange position of being a mature company in an industry that is still developing at breakneck speed. Given that California's lack of tradition or established values has been a factor in the propensity of its people to try new ideas, it will be interesting to see whether its deliberate acquisition of hagiographic baggage will slow it down. "There's no doubt that the meeting between Bob and Herb is legendary now", says Lasky. The money in the mugs, though, is a kind of self-deprecation, combined with youthful

Brook Byers:
has nurtured
31 biotech-
nology
ventures.



only do venture capitalists have to be more scientifically literate than most scientists, they have to talk the same language as the financiers — especially if they are asking for large sums to underwrite ventures that are speculative, by definition. "It's a very unusual profession", says Byers.

And as with many unusual professions, California provides a more sympathetic environment than perhaps anywhere else. "There are about 300 venture capital firms in the US", says Byers, "and half of them are in California."

Apart from the high concentration of fine science, venture capitalism took root on the West Coast because of attitude. "It's a different kind of investing", Byers explains. "It's an investing style that's very active, it's very committed, that requires a lot of passion and involvement. It's not passive. It's not negotiating the terms of the deal — that's really not done. The real risk in these companies is 'can you get the science to work?', it's not 'did you get 50 cents a share less price?' In the end all that matters is was it a really big idea, a major breakthrough." □

Standing on shaky ground

THE catastrophic magnitude-9 Alaska earthquake of 1964 helped usher in the new age of plate tectonics. It also prompted a westward migration of seismologists to USGS Menlo Park and the San Andreas fault. But even after 30 years, the underlying triggers of earthquakes remain obscure.

Bill Ellsworth of the

rocks so as to work out failure recurrence periods longer than human history.

Recurrence rates may help in predicting earthquakes, but can say nothing about the causes. This is why Ellsworth, his colleague Stephen Hickman and Stanford geophysicist Mark Zoback have put together a proposal to drill a hole 10,000 metres down into the San Andreas fault, to study its physics, mineralogy and mechanics.

They plan to use careful feasibility studies to select the best location for this hole, in which they would then conduct an extensive programme of geophysical measurements and sampling, before instrumenting the hole as a kind of deep fault 'observatory'. The work will build on the continuing German initiative to drill 10 km into the crust. More than a hundred people from several countries attended a workshop on the drilling project last December. The idea for an international collaboration is said really to have taken root.

One puzzle the project could solve arises from laboratory simulations of earthquakes. When scaled up to life size, the

In a state of shock

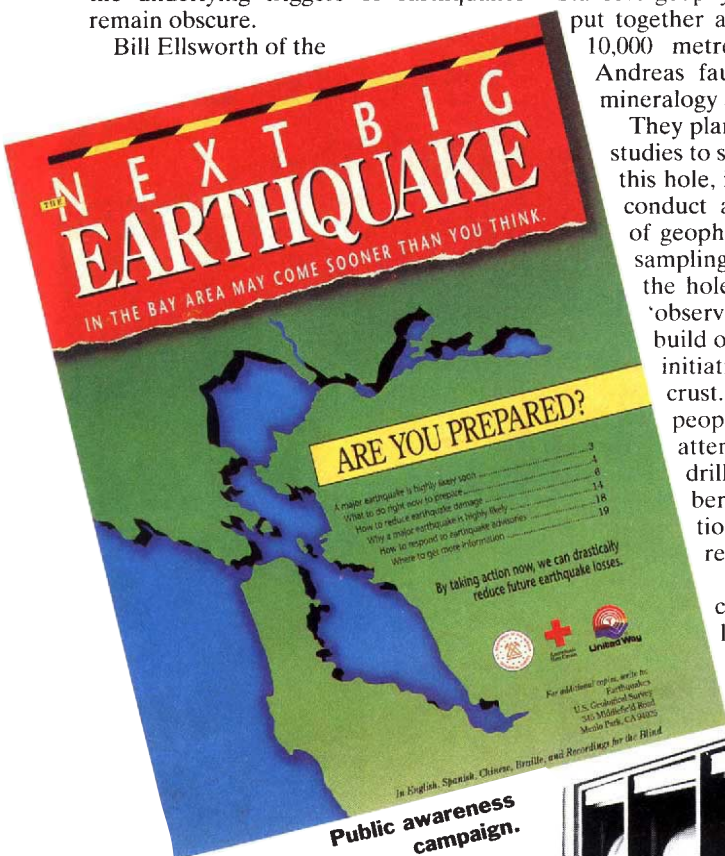
IN the next 25 years, the chance of a large earthquake (between magnitudes 7.5 and 8.5) happening on the San Andreas fault near Los Angeles is about 60 per cent — better than even. Such forecasts are the stock in trade of the Southern California Earthquake Center (SCEC), a body charged with informing the public about earthquake hazard, and coordinating research efforts into seismic research in southern California.

With the population rising rapidly in the area, seismologists are concerned about the increasing threat of earthquakes to human life. SCEC emerged from a workshop in which seismologists and geologists from seven campuses in southern California felt that they could achieve more together than apart — particularly in terms of funding. In 1991, SCEC was born as one of the National Science Foundation's Science and Technology Centers, with a modest fund of \$1.8 million a year for 5 years, with a possible extension to 11. The US Geological Survey (USGS) stepped in with another \$1.2 million a year, administered through its Pasadena office on Caltech campus.

Lucile Jones of USGS Pasadena takes communications with the public very seriously. Before SCEC was set up, she and her colleagues devised ways to calculate the short-term risk of an earthquake on the San Andreas fault (based on analyses of foreshocks), with graduated levels of alert, and protocols of what emergency services should expect to happen at each level.

At 7.30 p.m. one evening when she was at home, a report of a magnitude 4.6 earthquake came in from near the San Andreas fault near Palm Springs, providing an opportunity to rehearse disaster routines. Jones went back to the office to telephone her colleagues and the state emergency services, and was on the telephone when the magnitude 6 Joshua Tree earthquake happened. Husband and Caltech seismologist Egill Hauksson was at home when the earthquake struck. With no time to get a babysitter, he grabbed the kids and went back to work — only to meet a throng of media people anticipating the Big One on the San Andreas. Comforting a child in her arms and explaining earthquake hazard on television at the same time, Jones "went from being vaguely known to being a celebrity" at a stroke.

The media are, of course, central to this effort. At Caltech there is a media centre with seismometers ticking away, and television screens linked electronically to real-time earthquake monitors that display locations and magnitudes of

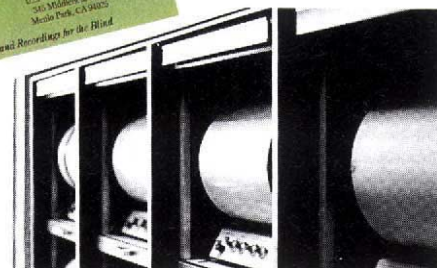


Public awareness campaign.

USGS is less concerned with the 'why' than the 'when'. From stress patterns and slip rates in particular fault segments, Ellsworth is sure that earthquakes have a history: many return, time and time again, to the same spot. The trick is finding out when.

The earthquake has not returned to the instrument-festooned Parkfield segment (yet), but other less well-publicized predictions have been uncannily successful. In 1990, for example, David Oppenheimer and colleagues from USGS surveyed a quiet section of the Calaveras fault near Gilroy (40 miles south of Menlo Park) where was an earthquake in 1949. Calculations of the speed at which the fault was moving suggested activity in the near future: there was, indeed, a magnitude-5.2 shock on 16 January 1993. Earthquakes are not entirely random.

Randomness may even be an illusion arising from the brevity of recorded history compared with the recurrence time of earthquakes, especially very large ones. Hence efforts to extend the record by trenching into fault zones and dating



Media-friendly seismographs at Caltech.

simulations suggest that earthquakes should generate a great deal of heat. But if the San Andreas fault, moving at 3–5 cm per year, generated a commensurate amount of heat, people would surely have detected it. "It strikes right to the heart of how earthquakes work", says Zoback. "If we don't know how the fault works, we don't know what to study in the laboratory and we don't know what to model theoretically."

Many believe that laboratory experiments are not representative of faulting along the San Andreas. It appears that major faults at plate boundaries are actually very weak, and possibly lubricated by highly pressurized fluids. It is "scary", says Zoback, that despite intensive study of earthquakes, we remain so ignorant of the fundamental processes that control faulting. □

earthquakes as they happen — which is constantly. This initiative is called CUBE (Caltech-USGS Broadcast of Epicenters).

Because of initiatives such as SCEC, “my guess is that 50 years from now, earthquakes will be much less of a problem for human life”, says David Jackson, professor of geophysics at the University of California, Los Angeles (UCLA). There will always be property damage, and part of SCEC’s job is to advise engineers on what they can get away with, rather than devoting enormous sums to proof against really big earthquakes that may not happen in the building’s projected lifetime. Nevertheless, money is a problem: “the cost of seismic upgrading right here on the UCLA campus during the 1990s is something very close to \$100 million”, says Jackson. Such figures make the \$3 million a year for the SCEC a price worth paying. □

SLAC

Ring the changes

My directions to SLAC (the Stanford Linear Accelerator Center) were faultless, and I arrived an hour early. Twenty billion years earlier, though, the infant Universe had not the benefits of SLAC’s public relations department, and somewhere along the line took a wrong turning: the result was a slight excess of regular matter over antimatter. The rest is history, and were it not for that excess, the Universe would be free of matter of any kind, and we would not be here to talk about it. It is embarrassing, therefore, that the Standard Model with which physicists like to explain everything cannot really explain the source of this initial discrepancy, called CP (charge-conjugation-times-parity) violation.

For something so fundamental, the only generally accepted experimental evidence for CP violation comes from the decay of a particle called the neutral K meson. Another design concerns another meson, the B meson. There are reasons for thinking that, every now and then, B and anti-B mesons decay in slightly different ways characteristic of CP violation. The problem is that such discrepancies would be tiny, the events rare, and that B mesons (discovered in 1977) are exotic to start with. A way to produce a lot of them would be to splurge beams of positrons and electrons together. If the beams were really intense, just now and then one of the results would be a particle called an upsilon, which decays into a B meson and its antiparticle, both of which would decay into a collection of other neutral and charged particles.

Close examination of the constituents in each case might reveal CP violation and, hopefully, something about its nature.

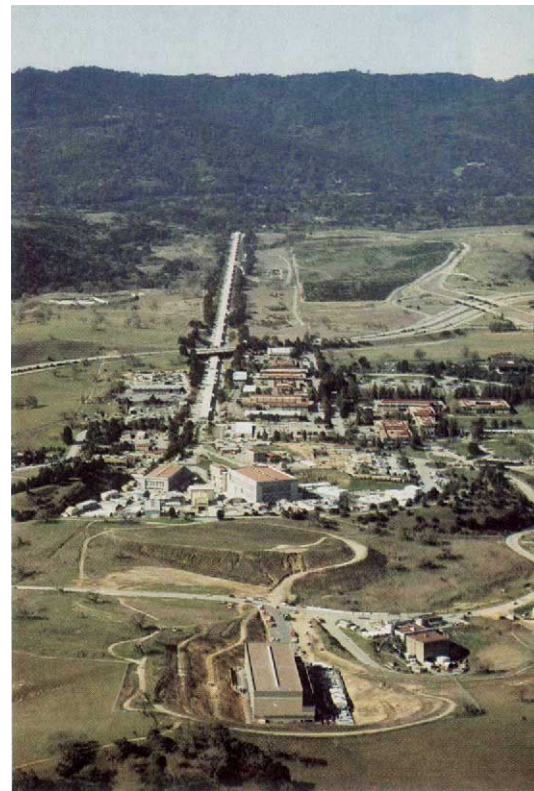
SLAC is ideally placed for such an experiment. It is a world leader in electron-positron beam technology, and could perform it with a (relatively) inexpensive add-on to an existing electron-positron storage ring. But there is not enough money to go round. In itself, that is not news, and the philosophical would heave a sigh and say that as the problem has been with us since the beginning of the Universe, a few more years will hardly matter. But history has conspired that SLAC as a whole will be in a perilous position were it not to secure funding for a B-factory — and soon.

It was not always thus. SLAC’s Director, Burton Richter, came to Stanford in 1956 to do physics with electron beams, the constant theme of his career: it was thus only natural for him to become involved in Stanford’s brand new linear electron accelerator from its inception. In 1968, just two years after construction was complete, researchers at SLAC found the first evidence for quarks. In 1971, work started on SPEAR, a 8-GeV electron-positron ring. In what became known as the ‘November revolution’ in 1974, SLAC researchers working on SPEAR found the J/ψ particle, indirect evidence for the charmed quark, work that led to a Nobel for Richter (shared with Sam Ting from MIT, who with his colleagues found the particle at Brookhaven). That Nobel year, 1976, SLAC researchers at SPEAR did it again, with the discovery of the τ lepton.

Then things started to unravel. A whole raft of information on the structure of matter has been prized from the 36-GeV PEP (positron-electron project) ring, started in 1976 and completed in 1980, but the top quark forever remained just out of reach. Since 1991, PEP has remained quiet, and may stay that way unless SLAC gets the go-ahead to upgrade it into a B-meson factory. A year earlier, 1990, the venerable SPEAR was converted to a synchrotron radiation facility that is being used for a myriad experiments — but not particle physics.

Then came the Stanford Linear Collider (SLC), the electron-positron collider that raced against the LEP machine at CERN to produce large numbers of Z^0 bosons and thus constrain the number of particle ‘families’ in nature to three. Even though SLC can do things that LEP can’t, such as polarizing its beams of electrons to show that Z^0 s are produced, preferentially, in ‘left-handed’ beams, it has never matched LEP for sheer numbers of particles.

Posterity will hail the SLC as the pioneer of a new age of linear colliders. “Everyone agrees that storage-ring technology has finished with LEP,” says



SLAC: still with a future.

Richter. “The only way to go to higher energies with the electron-positron system is with the linear collider. And there will be, some time in the early 2000s, a linear collider at high energy — and this one is going to be an international collaboration”. Indeed plans for a more powerful linear collider are being drawn up, involving researchers from the US, Europe, Japan and Russia.

Such an ambitious project seems some years from fruition, and in the meantime SLAC must get by on R&D and synchrotron experiments. There are worries that without the immediate charge of cutting-edge particle physics offered by the B factory, SLAC is left exposed to cuts. A plan to streamline SLAC’s operation so that the B-factory could be fitted into the operating budget, provided that the rest of SLAC’s operations remained funded, met with the response that were instituted, the saving could be devoted to work at other DoE laboratories — and SLAC would have to wait for its B-factory, possibly until 1995. B-factories elsewhere would have had the chance to catch up on SLAC’s lead.

But prospects at present seem slightly brighter than what was always a worst-case scenario. The end of the Cold War has allowed the DoE to be more flexible in its budgeting, and provision for the B-factory appeared in budget plans drawn up by the Bush administration. Whether the Clinton administration adopts the B-factory remains to be seen, but opinion within SLAC seems optimistic that funding will be appropriated. □

Talk of the town

CIRCUMSTANCES of a cruel and unusual kind must prevail before radio commercials mention the word 'recession'. In downtown San Diego, radio advertisements for sales at knockdown prices were interrupted by the business news: a local company had received approval from the Food and Drug Administration to make and sell a diagnostic kit for brucellosis. The slump in defence spending has knocked the Navy town of San Diego for six — but if you're in biotechnology, it's never been better.

The biotechnology community is largely concentrated in La Jolla, just to the north of the city itself. Several research institutions — most notably the Salk, the Scripps Research Institute and the University of California, San Diego (UCSD) have spawned or attracted more than 150 biotechnology companies, all within what is effectively a campus no more than five miles across. Much of the federal funding for research in the area comes from the National Institutes of Health (NIH), which have only just woken up to the potential for tax dollars to go astray in the labyrinthine business connections that run up and down North Torrey Pines Road.

The heat is currently turned on Scripps, and its president, biochemist Richard Lerner, who is supremely good at attracting industrial sponsorship: a talent that attracts both admiration and envy. Scripps currently receives \$10 million a year (about a twelfth of its budget) from the drug company Johnson & Johnson (J&J) in exchange for first rights to develop discoveries made by Scripps researchers. The contract with J&J will to be replaced in 1997 with an agreement with the Swiss firm Sandoz. Over a 10-year period, Sandoz will give Scripps \$300 million for the right of first refusal for its products. Given that federal agencies, notably NIH, will add \$1 billion in grants over the same period, concern has been expressed that a foreign company is getting first bite at a \$1.3-billion-worth of publicly funded research for an investment of \$300 million.

Thanks to coverage by business reporter Craig Rose of the *San Diego Union-Tribune*, the Scripps-Sandoz deal (with the added spice of a congressional investigation into the lack of NIH oversight of the deal and others like it) is the talk of the town. Lerner is unrepentant: "our responsibility is to discover things that allow drugs to be made, and we need money to do that — and that's our pure and simple mission. After that, Congress and the pharmaceutical companies can argue with each other about to what extent the taxpayers are paid, and to what extent they deserve a tax break. We

don't know anything about that, and we don't want into that issue. We are charged with discovery, and that's what we do for a living." The J&J arrangement had modest beginnings, says Lerner's former Scripps colleague and departmental chairman David Katz. But the final terms were rather more inclusive than originally foreseen, encompassing the work of some Scripps researchers allegedly without their full knowledge. "Rather than give implied consent by remaining, when I knew what appeared to be happening — and I disagreed with it", says Katz; "it was better for me to move on." Katz now heads the Medical Biology Institute (MBI) in La Jolla, as well as the for-profit company Lidak Pharmaceuticals, which has rights to MBI's technology.

Representative Ron Wyden (Democrat, Oregon) has got the bit between his teeth about the NIH's apparent inability to keep track of the funds it awards to institutes with industrial links. He threatened Scripps with withdrawal of NIH funding if it did not cooperate with

inquiries (it is cooperating, if after initial reluctance), and has questioned NIH about oversight for funding of MBI and other San-Diego-based companies. "These strings [between industry and research] are more imaginary than real", says Lerner: "the pharmaceutical company cannot tell the investigators what to do."

Although just two minutes' drive down the coast, researchers at the Salk Institute adopt a completely different stance. "I'm not clear myself on how we relate to biotechnology firms, individually or collectively. It's an area of considerable stress" says Salk's new president, molecular epidemiologist Brian Henderson. "We are concerned about conflict-of-interest issues, the commercialization issue; how do we benefit as an institution? How do individuals benefit, and how do we do that in the face of maintaining an independent research activity that's not driven by product development *per se*, but driven by ideas? I think it's a difficult road." Henderson would stay with the Salk's self-confessed "conservative" approach until he can identify a regimen "that would benefit us but not tie us to anybody else's agenda". □

At the zoo ...

EVERY animal is a dark continent under the skin. To those whose knowledge of mammals begins and ends with the white rat, it should be sobering that only half the species of mammals have ever been karyotyped, let alone more deeply investigated.



John Phillips and friend.

For the two dozen researchers at the Center for Reproduction of Endangered Species (CRES) at San Diego Zoo, the generalizations espoused by other biologists are laughable. John Phillips, deputy director of the centre, tells how a grant proposal to study a feature of lizard physiology drew the opinion from a referee that he could find the same things more cheaply in a white rat.

At the coalface of biodiversity, where snap inferences about an alien physiology can mean the death of a species, people are used to surprises. Virologist Mike Worley and his colleagues, for example, have noticed signs of liver disease in black rhi-

nos, with signs of what in other animals would indicate a kind of hepatitis. The rhinos are seropositive, but no viruses have yet been found; where have they gone? Is it the weird glucose metabolism of the rhino that makes it acutely sensitive to oxidative stress? No shotgun can bring down a rhino as surely as a burst of explosive haemolysis.

And why, asks geneticist Oliver Ryder somewhat rhetorically, do the zoo's Vietnamese douc langurs have chromosomal translocations that cause reproductive failure? Is it because their home forests were once sprayed with Agent Orange? Or are zoo researchers mistakenly trying to impose cross-specific misalliance? To help

answer such questions, CRES has a 'frozen zoo' of cell-culture materials from 300 different mammalian species, the biggest anywhere.

The answers underpin CRES's chief objective, to understand a species sufficiently well that it breeds in captivity and can be reintroduced into the wild. The captive-breeding programme for the Californian condor has been a great success. The relict population of about 20 birds has now been built up to around 70 at the zoo's Wild Animal Park. But the zoo lives and learns: nothing can stop a condor from drinking from a roadside puddle polluted with antifreeze, with fatal results. □

A night on Palomar Mountain

A SIGN reading "SCHOOL" is the unexpected find at the end of a hair-raising climb to 5,000 feet on a road more suited to a jeep than a rented automatic. It deflates any sense of achievement at having reached the summit. This is the community of Palomar Mountain, just like any other small town, except that the residents ranch telescopes and look after the people who come to use them.

A source of quiet pride to the residents is the giant 200-inch Hale telescope, until relatively recently the biggest and most powerful telescope in the world.

Despite the encroachment of city lights, and the construction of bigger telescopes in darker places, the Hale is still a magnet for astronomers. Why?

First, it is simply a joy to use. The spacious open design means that it is easy to install, adjust and upgrade instrumentation packages at the two main foci. Combined with this versatility, the instrument is engineered with military precision and with durability in mind. That it looks like a battleship is no coincidence — the chief engineer was a naval architect on special leave from the US Navy.

Second, cutting-edge observational astronomy does not always require the biggest telescopes. "It would be criminal to have all telescopes in the 400-inch class" says Robert Brucato, Assistant Director of the Observatory. As well as the Hale, Palomar has a 60-inch, a 48-inch and an 18-inch telescope, all in constant use. The Second Palomar Sky Survey, now half complete, is compiled with the 48-inch Oschin Telescope. The 18-inch instrument is currently being used to monitor Earth-crossing asteroids.

Not that the Hale, this Rolls-Royce of telescopes, is without its problems.

The observatory is run by Caltech; for many years, time on the Hale has been shared with the Carnegie Institution and Cornell, which each have a quarter share. What was once a gentleman's agreement is now a financial arrangement, with the Carnegie and Cornell contributing to the operating costs and the development of new instrumentation. But the Carnegie has chosen not to renew its contract in 1994, and will concentrate its efforts on the Las Campanas observatory in Chile. Caltech now has to decide whether to take up the slack itself or approach another institution.

Should Caltech decide to offer the time elsewhere, how will it sell the idea of Palomar against the competition of newer telescopes in places less prone to light-pollution? The demand for time on big telescopes is so great that a quarter-share of the Hale will be an attractive proposition.

Life in the small town of Palomar

Mountain is thus likely to continue much as it has for the past 50 years. Robert Thicksten is the Site Superintendent of the Observatory. "I sometimes call him the Mayor", says Brucato, because in addition to keeping the telescopes in working order, he and his staff fix roofs, mend broken cables, ensure that the snow-ploughs keep the roads clear and generally keep the town's amenities in good shape.

Once they get too old for the elementary school (faculty of one), Palomar kids are bussed down the mountain to the local high school (weather permitting). Luckily for them, there is an alternative

THE SALK

Oxbridge-on-Sea

WERE one to transplant an Oxford or Cambridge college to the Pacific seashore, it would look like the Salk. The building itself fits the bill and the collegiate atmosphere is as pervasive inside as out. The 45 faculty prize academic excellence above all, and the result is that the Salk has slightly fewer financial resources than required to keep it hanging on by its fingernails. Yet Salk publications have four times the citation impact of those from the flush and populous Scripps Institute.

Far from moaning about their impoverishment, Salk researchers see their institute's limitations as the key to their success. For one thing, the entire faculty can fit into a classroom, which allows for an uniquely democratic form of internal government.

Second, almost all the Salk's funds come from federal grants. The institute guarantees its faculty a salary, from which the value of current grants is deducted. In practice, it would be unable



Not biggest, but one of the best.

route down the mountain — longer, but easier on the gear-shift. □

Salkitecture

THE cover to this week's issue is an example of the Salk's striking architecture. Just before Christmas 1959, Jonas Salk ferreted bohemian architect Louis Kahn from his office at the University of Pennsylvania. Salk, famous for the eponymous polio vaccine, had had a vision of a research institute with a creative environment "in which a Picasso would be comfortable working". Kahn was an Estonian emigré who is said to have sustained a varied sex life while living with his mother-in-law, and liked nothing better than to watch women roller-skating. The two hit it off instantly. In 1960, Salk found the site, Torrey Pines Mesa, a clifftop on the edge of the Pacific. With nothing more than a pledge from the March of Dimes Birth Defects Foundation, Salk and Kahn asked the City of San Diego for the site. After a referendum, it agreed, and in 1965, the institute opened its doors. It has since been judged one of America's ten most distinguished buildings, and has won the American Institute of Architectures' 25-year award.

to honour this guarantee were more than a few researchers to lose their grants, but the rigorously selective recruitment policy ensures that successful applicants are of the kind likely to bring generous grants with them. Such risk is courted by the faculty as a way to keep their research up to scratch.

The endowment of \$20 million is far smaller than the institute would like, for all its ideals. The relationship with its commercial subsidiary, SIBIA, is born of necessity rather than desire, and will end as soon as the company goes public and can yield a return to shore up the endowment.

All in all, the collegiate philosophy here is more ascetic than the all-guns-blazing entrepreneurial approach seen, for example, at Scripps. One might say that it is more European than American in character: this is hardly surprising, in that many of the original and early fellows came here from Europe. "When I first came here in 1970", says neurobiologist Steve Heinemann, "the fellows ran the place, and it had a very European flavour."

But expansion is in the air, and by the time you read this, the foundations should be laid for a new building. The Salk badly needs a transgenic facility, more laboratory space and an auditorium. Heinemann is worried that the extension could dilute the advantages of smallness. Papers with big citation impacts, he feels, come from small places. Virologist Inder Verma is less worried, although he says that "we are not used to growing by quantum leaps". Biochemist Tony Hunter is more sanguine, in that meetings held in the auditorium could help to dispel the institute's somewhat aloof image: "we've had that reputation of not talking to the community" he says. □

April come she will

"THE perception of injustice is one of the most powerful stimulants of anger, violence and aggression in humans," says Seymour Feshbach, a student of human aggressive behaviour and professor of psychology at the University of California, Los Angeles (UCLA). He adds that this human trait is one of the most pronounced differences between human and animal aggression.

Feshbach has a natural laboratory on his doorstep: south-central Los Angeles. Last April (a year ago), this was the scene of abandoned affray, some of it broadcast live on television, following the beating of a black motorist called Rodney King by several police officers.

A year later, south-central is still a riot waiting to happen. The outcome of two trials, one the federal trial of Rodney King's tormentors on somewhat abstract charges of restricting King's 'civil liberties' and the court appearance of blacks charged with dragging a white truck driver from his cab and beating him almost to death, could provide the spark.

Feshbach was well placed to co-ordinate UCLA's response to April's spasms. Following a statement by the Chancellor of UCLA's commitment to healing the wounds, Feshbach was appointed as the Chancellor's special assistant to co-ordinate long-term links between campus and city.

In essence, Feshbach's task is to keep track of, and if possible to integrate, the many independent and disparate university social programmes, to raise money for them and to link them with UCLA's academic mission. Lessons learned can add depth to academic programmes, in that students of social sciences can receive what Feshbach calls "meaningful field experience" that is not available elsewhere.

To be fair, UCLA's professional schools such as social welfare, architecture and public health have had a continuing involvement with the city which, after all, provides its research material. At the other end of the spectrum, the UCLA student body has a voluntary and active Community Service Commission (CSC) which coordinates outreach programmes, and was doing so before the riots. The CSC is beyond Feshbach's remit, although he now has funds to help it if needed.

If the variety of post-riot initiatives has a common theme, it is the facility of university people to devote to the city's problems time and analytical skills that the residents lack. More than 600 faculty members have volunteered to provide specialist services (such as urban planning, architectural drawing, counselling, business and legal advice) through the

university's Office of Community and Governmental Relations.

At the same time, community leaders visit UCLA for periods of between six and 12 weeks as part of the Community Scholars Program, to learn how to go about such things as writing letters to secure funding for a community organization.

Yet for all this activity, many of the residents in south-central are unaware even of the existence of UCLA. Unlike the University of Southern California, which is right in the thick of it, UCLA might as well be on another planet. So there are moves to set up a social services centre in south-central, a cross between a mission and a field station.

Those who know about UCLA, on the other hand, are inclined to be suspicious of its motives. After the Watts riots 30 years ago, people felt that they were being exploited as a laboratory for the benefit of scholars. Yet there is also a

feeling that the university, with its resources and skills, will be able to solve all LA's problems. So, beneath the cynicism and criticism, there are "unrealistic fantasies" of what the university can do: UCLA must be careful not to make promises that it cannot keep.

Relations between the university and the police are only just beginning. Faculty are starting to appear at the police colleges, talking about such matters as ethnic diversity. Just over half the students at UCLA come from LA, and there is no single ethnic majority. About a third are whites, a third Asian, and most of the rest are Hispanics. Blacks constitute only 14-15 per cent, and are a declining presence both on campus and in the city.

Where are they going? Feshbach says, "they are either leaving the city and going to other parts of southern California, some are returning to the South, and others are getting killed." Shooting constitutes the single biggest cause of death in young black men, and it is having an effect on the demography, even if to keep blacks away from LA. □

LOS ANGELES MUSEUM

A circle of fire

SOME of the T-shirts on sale at the George C. Page Museum, just off Wilshire Boulevard, Los Angeles, will be advertised as pre-washed. One of this winter's downpours, exceptional after six years of drought, found its way into a basement stockroom, soaking the

found all the holes in museum research, poorly funded at the best of times, and has given it a thorough soaking. In Los Angeles, social problems add to the funding crisis. During last April's civil unrest, says Black, Exposition Park was "ringed in fire. No-one came in here, and there was no damage, but it leaves a perception in the public mind. 'Is that a place I really want to go?'"

The Los Angeles County Museum began in happier times, in 1913. One of its tasks then was to oversee the excavation of the Rancho La Brea Tar Pits, remarkable asphalt seeps that have yielded thousands of bones of Pleistocene animals. For around 40,000 years, bison, sabre-tooth cats, wolves and mastodons roamed what is now Rodeo Drive, and some of them

became ensnared in the tar pits.

It is easy to see why. Just one tar-pit remains (complete with statues of mired mammoths), with a thin sheen of surface water that looks, deceptively, deeper and more drinkable than it is. The sprawl of downtown LA engulfed the site sometime in the 1930s, and in 1977, the George C. Page Museum was erected on the site to display the finds. But Black,



Mammoths in the tar pits.

merchandise for the museum shop. Craig Black, director of the Los Angeles County Museum, drove to Beverly Hills through the rain from the main museum in Exposition Park — a green square amid the seedy south-central district — to inspect the damage, which he was thankful to find minimal. But rainfall is the least of his worries.

Like the rainwater, the recession has

IBM

even though a palaeontologist by training, has looked at very few fossils recently. "I spend all my time raising money" he says. Sixty per cent of the museum's \$21-million annual budget (for the main site, the Page and a small history museum out of town) comes from Los Angeles County. But these funds are discretionary, and are increasingly under pressure from the load of mandatory spending on essential services to which the County is committed. The rest comes from earned income, gate receipts, shop revenues and fundraising.

The crunch is likely to continue, so the long-term health of the museum will depend on the private sector. In the short term, decline in county funding has forced Black to make some hard decisions. This year, he has had to cut \$2.2 million from his budget — well over 10 per cent. Forty-seven positions have been lost by early retirement or redundancy, almost a third of the total staff of 157. Of 42 PhD-grade researchers, eight have been laid off.

Black's mission has been to build the museum's endowment, worth just \$10 million when he moved in; now it is worth \$25 million. The museum harvests \$0.5 million of the yield each year; the rest is ploughed back. The museum knows that this thrifty policy might have to change if matters take a leaner turn. Black is meanwhile negotiating three endowed curatorships at \$1 million each. That may sound hopeful, but it is only a small part of the endowment of \$150–\$200 million the museum would need to become self-supporting. Miracles, they say, take a little longer than the merely impossible.

All the while, Black has to contend with the one question raised by prospective sponsors: if the museum is supported by the County, why should they make any contribution over and above what they pay in taxes? Large corporations, luckily, are keen to provide sponsorship, although these days they prefer to be associated with inner-city education and outreach programmes than blockbusting exhibitions. This is perhaps a blessing. The main museum sits uneasily in its inner-city location, and may depend on good relations with the community for survival. Since the civil unrest, the recession has combined with the local situation to pull gate receipts down by 15 per cent.

Nevertheless, there are good relationships with schools, who are not put off by the museum's scholarly image as much as the casual visitor. And an 'apprentice' programme that brings in high-school students to work on research problems on Saturdays has attracted ethnic minorities and, in turn, favourable interest from sponsors. This programme could expand considerably if there were money to pay for the necessary staff. □

Disk drives, hard times

IN the space of two years in the late 1980s, researchers at IBM walked away with Nobel prizes for the invention of the scanning tunnelling microscope, and the discovery of high-temperature ceramic superconductors. But within five years, IBM reported the biggest corporate deficit (\$5 billion) ever. The cutback in research expenditure this year is expected to be about 10%.

This is curious given that IBM has been a part of computing since the very beginning. IBM's roots in California go back 40 years — the magnetic disk-drive inside every desktop computer was invented here, at IBM in San José. The first research laboratory in town was a small storefront operation — four buildings on, it is a stylish complex in the green hills out of town. The Almadén laboratory now houses about 650 of the 3,000 people in the laboratories of IBM Research worldwide.

IBM research has traditionally been

curiosity-driven, with just half an eye on product development. So although good at winning Nobel prizes, the company has had more of a problem in getting its



Almadén: IBM's corporate cutbacks must take a toll

ideas into products. Nevertheless, commercial products do come out of such arcana: the STM has proved a very useful research and industrial tool, and fractal geometry (invented by Benoit Mandelbrot, an IBM researcher) is finding its way into such practical things as data-compression algorithms. □

EXO BIOLOGY

Recipes for primaevial soup

EXO BIOLOGISTS are scientists in search of a discipline. Or are they? Exobiology is really a catch-all term for people of diverse backgrounds, connected by a common interest in the origin of life. More than a dozen physicists, chemists, biologists and geologists work at the NASA Ames Research Center under the exobiology umbrella.

One of them is organic chemist Sherwood Chang, who was recruited by NASA Ames while at Stanford in 1967.

That NASA should devote resources to exobiology is a natural extension of its mission. "Life is clearly a planetary phenomenon", says Chang. "Its origin and evolution, at least on Earth, has been intimately connected with the history of the planet itself, and the question of extraterrestrial life therefore must also be directly connected to the origin and the evolution of other planets. If we're to understand how life began on Earth it behoves us to ask similar questions of a planet like Mars, of planets like Venus and other planets accessible to study in the Solar System."

Exobiologists benefit from the results of NASA's space missions, but also contribute their own ideas to the effort. "NASA needs the rationale for the science that it does. Scientists who provide that rationale need NASA's missions, so,

yes, it's a two-way street."

Until recently, however, only relatively mature researchers have been able to enter a field thought too controversial for postdocs to risk. This could change, thanks to a new NASA programme, instituted two years ago, to award \$1 million a year for five years to an institution judged fittest to teach a programme in exobiology. The joint winners were a consortium of the University of California, San Diego, Scripps and the Salk Institute, which pooled their resources to formulate a program. Eventually, this San Diego nucleus will produce a cadre of young exobiologists.

Almost half the NASA-supported exobiologists work in California. Is this because California treats with sympathy matters that others find outlandish? It could be, simply, that many of the advocates of work on the origin of life have been associated with California at some time or other. Stanley Miller (UC San Diego), Leslie Orgel (Salk), Melvin Calvin (Berkeley) and Joshua Lederberg (Stanford) spring to mind, among others. But it may be no coincidence that burgeoning interest in this novel research area matched the rapid growth of higher education in California in the 1960s. Or could it be because, as Chang says, California is "on the edge of things"? □

The mackerel experiment

CALTECH opened its doors on 2 November 1891, when a man named Amos G. Throop decided to do something more interesting after lunch than plant potatoes. Instead, he founded a school of arts and crafts. The story could have ended then and there but for the arrival, in 1907, of a new member of the board of trustees: the astronomer, George Ellery Hale. In 1903, Hale came to Pasadena from the University of Chicago, to look for somewhere to put his 60-inch telescope. He settled on nearby Mount Wilson.

By 1920 he had persuaded chemist Arthur Noyes (former president of MIT, the Massachusetts Institute of Technology) and physicist Robert Millikan (Professor of Physics at the University of Chicago, Hale's old school) to help him transform Throop into MIT on the West Coast. In 1920, Throop became the California Institute of Technology.

Today, the professorial faculty is around 300, with around 1,100 graduate students and 900 undergraduates. Like the Salk, it has remained small by choice.

This policy has worked, if the large number of Caltech Nobel Laureates (21) is a measure.

About half its \$253-million budget comes from federal sources directly. The other half comes from industrial support, channelled through Caltech's Office of Sponsored Research (OSR). Like the Salk, Caltech takes a tough line to preserve its independence, and insists that the publication of industrially sponsored research is not delayed by patent review. But Caltech has been running for three times longer than the Salk and has, therefore, had the chance to accrete an endowment of \$580 million. The faculty of this private school is among the best paid in the country.

It hasn't always been such a bed of roses. For example, it is hard to understand, when women had attended Throop from the first, why Caltech decided to bar them after 1910. Another 60 years were to pass before that policy was revoked. It is even harder to understand why, in what must rank as one of Caltech's lesser known achievements,

the Class of 1937 sought to discover the distance between Caltech and Pasadena City Hall in terms of the number of mackerel, laid end to end (5,678). In current circumstances, though, it is easier to understand why, in the Depression, Millikan (by then the Chairman of Caltech's Executive Council) asked faculty to take a salary cut of 10 per cent — twice that faced by UC faculty today. (They agreed.)

Such events though, account for Caltech's character, typified by an initiative that stands somewhere between what staid folk would consider possible, and the mackerel experiment: that is, LIGO — the Laser Interferometer Gravitational-Wave Observatory (see photo), a collaboration between Caltech and MIT. LIGO is designed to observe cosmic events inaccessible to the electromagnetic spectrum, such as the effect of supernovae and black holes on the space-time continuum.

The director of the LIGO project is Rochus "Robbie" Vogt, professor of physics and a member of the faculty for more than

30 years. Vogt's scientific team currently consists of about 40 people at Caltech and MIT, and construction has begun at the two sites. One is a former nuclear reservation in Washington State, the other is near Baton Rouge, Louisiana. By the time it is finished, LIGO will have cost about \$250 million: the NSF has granted \$19.1 million for the initial design and evaluation phases, and at the time of writing Vogt awaits another tranche of a similar amount, so the bulldozers can move in.

Importantly, LIGO only seems like "Big Science" (on a CERN or SSC scale) because of the immense technical problems involved. But these are of such consequence that, for LIGO to work at all, the team must be small enough for each member to have a good idea of what everyone else is doing. "It is vitally important that the LIGO team be a tightly knit, fully integrated and rather Renaissance kind of team," says Vogt, "where everybody is strong in something but knowledgeable in everything else. This is a fun project for a physicist." □

LAWRENCE LIVERMORE

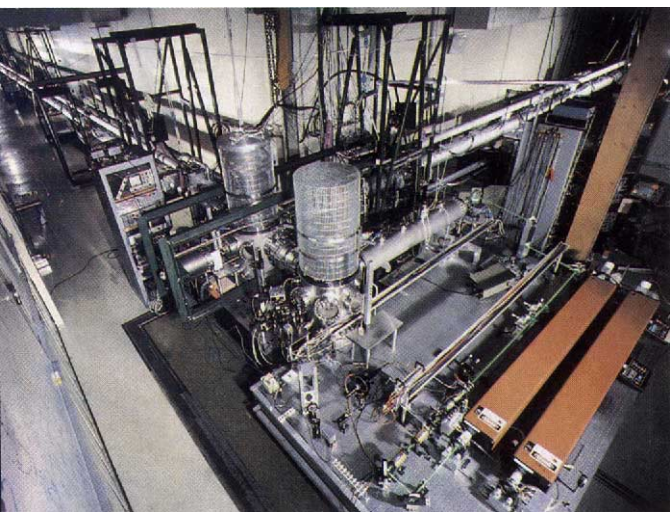
Beating around the bomb

ARRIVE as early as possible at a nuclear weapons laboratory. The time saved is useful for delays in the Pass Office. But after the required picture-taking and form-filling, I held the door open for a party of entering Russians. Such is the change at the Lawrence Livermore National Laboratory (LLNL).

Originally meant to service a programme of research into nuclear weapons and thermonuclear fusion, the laboratory has branched into almost every kind of applied science, and weapons research now accounts for less than 30 per cent. Environmental sciences, in contrast, has grown by 50 per cent per year, from about \$25 million annually in the late 1980s to almost \$200 million today.

Livermore was founded by Edward O. Lawrence in 1952 as a rival to the Los Alamos National Laboratory in New Mexico, which had been set up in 1943 as part of the Manhattan Project. The University of California administrators both, as well as the smaller Lawrence Berkeley Laboratory, on behalf of the Department of Energy. LLNL is the largest of the three, and consumes slightly more than half their combined budget of \$2.5 billion.

Most of the civil research here can trace its roots, ultimately, to the weapons programme or to the originally small component of the programme devoted to fusion energy research. Deeper still lies Lawrence's conception, that scientists



When complete, LIGO will consist of two identical, L-shaped buildings. In each observatory, each arm of the L will house a 2.5-km-long, 4-foot-diameter evacuated pipe, at the end of which will hang a weight adorned with highly reflective mirrors. Other weights will hang at the vertex. Highly stable, pure beams of laser light fired from the vertex along the arms to the suspended weights will be reflected back to the vertex, where they will interfere. The phase of each beam is reversed with respect to the other, so in normal circumstances, they will cancel out and no interference pattern will be observed. But if a gravitational wave happens to pass through the site, the suspended weights in one arm will be forced apart by a minuscule amount, whereas those in the orthogonal arm will be drawn together. This will change the phase relationships of the laser beams, and an interference pattern will emerge. The device is big enough to record variations in distance as small as 10^{-15} mm, a distance equivalent to 10^{-6} the diameter of a hydrogen atom. Two observatories will be needed to verify that the signal detected by one of them comes from space, and is not the result of local disturbances such as small earthquakes.

First contact

THOUGHTS of life in other worlds have motivated Frank Drake since boyhood. As a young radioastronomer, he felt that radio might prove the medium of first contact. One night, all alone with a radiotelescope, he detected what seemed to be a purposeful signal from the Pleiades. He reminisces that "it creates a very strong and unusual emotion to think that you may be in contact with another civilization". That signal was a false alarm, "but nevertheless, the seeds had been planted".

Since its beginnings in the 1950s, improved technology has made SETI (the Search for Extraterrestrial Intelligence) more respectable, and Harvard, UC Berkeley, Ohio State University and NASA (Ames and JPL) now have SETI programmes.

Drake is on the NASA Ames team, and is also president of the SETI Institute, a nonprofit corporation that works as a contractor to NASA to undertake the actual work. The SETI project itself is a \$100-million programme over ten years (now in its third year) to develop high-performance computer chips that function as multichannel radio receivers. The system now working can monitor 15 million channels simultaneously.

No amount of technology can second-guess extraterrestrial intentions and tell astronomers where they should point their telescopes. The right strategy, therefore, is to look at the nearer stars, because the signals may be the loudest. On the other hand, some may broadcast powerful signals from further away, in which case the nearer stars are no help. As a compromise, the NASA SETI project (or the High Resolution Microwave Survey, as it is to become known) based at Ames is scanning the 1,000 nearest stars, and the complementary search, based at JPL, is looking at the whole sky to pick out the loudest sources.

The first two months of listening have picked up 15 false alarms. Should SETI strike lucky, other telescopes would be brought in to verify any signal, and public announcement would be immediate. What would be its effect on the public? "I think it will cause great excitement, great elation in the world, and will lead — in time — to a great explosion in knowledge and understanding of our civilization, and a very rapidly changing attitude towards ourselves and our future", says Drake, who dismisses the fears of some that contact would have adverse consequences. Interstellar distances are a barrier to exploitation of the Earth from elsewhere, and "if we don't like what we're hearing, we just pull the plug. The fear that these other cultures will turn us into slaves is just science fiction." □



"A square mile of pure science", runs the Livermore's own caption to this aerial view.

should be able to dream up ambitious programmes and have the engineering expertise available to build big.

For example, the heart of the inertial confinement fusion programme is a gigantic laser called NOVA. The idea is to generate and focus a beam powerful enough to crush atoms together.

And the aptly named MACHO project is part of a collaboration to hunt for dark matter, on the hypothesis that it consists of invisible 'brown dwarfs' in the haloes of galaxies such as our own (MACHO stands for Massive Compact Halo Object). The brown dwarfs in the halo of the Milky Way would be detected from the lensing effects of their gravity fields on the brightness of stars in the Magellanic Clouds. Such events are likely to be extremely rare. The MACHO approach, therefore, is to measure the brightness of millions of stars at once, using the largest CCD cameras ever built, with the gigantic amounts of data processed by some of the fastest computers. MACHO has acquired more photometric data than have been gathered in the entire history of astronomy. And it's still only in its engineering phase.

Researchers on the Electron Beam Ion Trap (EBIT) can pluck electrons from atoms like berries from a bush. Work on the behaviour of highly ionized species (the most egregious is a helium-like uranium cation, U^{90+}) is adding a third dimension to the periodic table.

Livermore is the home of the DoE's contribution to the Human Genome Pro-

ject. This seems strange, until one realizes that LLNL has (naturally) among the most powerful gene sequencing technology to be found anywhere. Indeed, biological research is a valuable spinoff from other areas of research here, notably the laser programme. LLNL has led the world in the handling of X rays. It is now possible to reflect and refract X rays (almost) as easily as visible light. Thanks to X-ray lithography, it is now possible to cram a gigabit of memory onto one chip. Thanks to X-ray holography and laser microscopy, it is (or soon will be) possible to image the structures of proteins doing their thing *au naturel*, in living cells, in real time (see *Science* 258, 269–271; 1992). But lest we forget the joy of sheer power, the most powerful laser in the world is a petawatt

laser hardly larger than a benchtop. (A petawatt is equivalent to a billion megawatts.) On an even smaller scale, it is possible to cram 20,000 highly efficient diode lasers into a space the size of a gish postage stamp to yield 4,000 watts of laser power that can be used to pump larger lasers.

Much of this work would not have been possible but for a small part of LLNL's operation, the Laboratory-Directed Research and Development Program (LDRD). This exists to review, select and sponsor relatively small, speculative projects dreamed up by LLNL's research staff.

In the old days, the relatively few research programmes were funded *en bloc*, a policy that left plenty of flexibility for the discretionary funding of smaller, spin-off projects. This tendency grew until in 1970 such funding accounted for 15 per cent of the laboratory's budget. But with the passing of time, the same funds had to support an increasing diversity of major programmes, leaving less room for curiosity-driven research. It would probably have become extinct but for a deliberate policy decision in 1985 to allocate 2 per cent of the annual budget to what is now the LDRD. The proportion is now 6 per cent; the upshot is that the LDRD has \$59 million to fund its roster of about 140 projects to the tune of between \$50,000 and \$3 million each. In a way, it is depressing that the funding of serendipity needs a separate, conscious effort. □

Appetite for destruction

CALIFORNIANS worried about unemployment and social unrest can always find solace in drink. But the news is no rosier here: a new strain of the vine-loving louse phylloxera (*Dactulosphaira vitifoliae*) is munching its way through hitherto resistant rootstocks.

Phylloxera is native to North America east of the Rocky Mountains, and has been living in native vines since time immemorial. It turned up in Europe in the 1860s and laid vineyards to waste until growers learned how to graft vines onto pest-resistant rootstocks. It was found in California at about the same time, in vineyards that grew imported European varieties. The Californian wine industry was then in its infancy.

A rootstock called AXR#1 was found to be especially resistant. Researchers at the University of California, with the co-operation of local growers, planted AXR#1 in 1903 and continued trials of AXR#1 until 1958, without noticeable loss of phylloxera resistance. This is remarkable, considering that the resistance of AXR#1 grown in Europe in the 1870s wore off during the First World War.

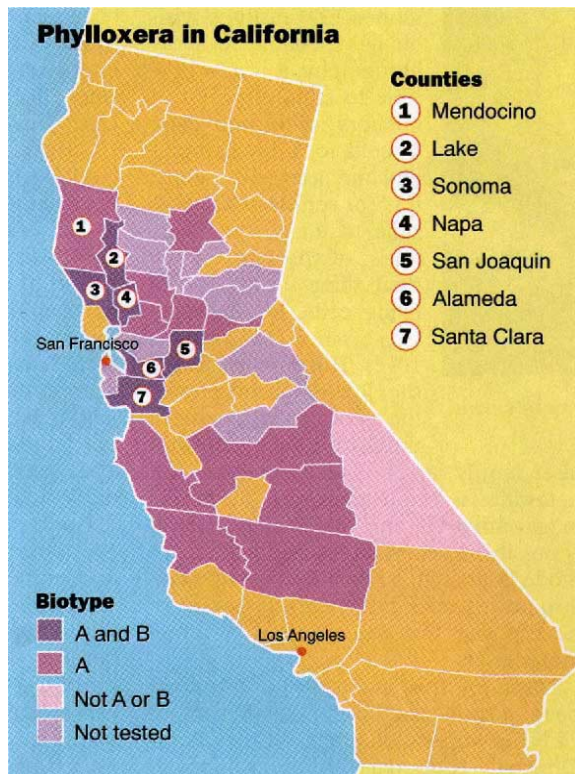
But at more than 70 years old, never a whisper of trouble, the continued health of AXR#1 seemed too good to last. In 1982, Jeffrey Granett, professor of entomology at UC Davis, and his colleagues found a strain of phylloxera in Napa and Sonoma counties, the heartland of the California wine region, that lived quite happily on AXR#1 roots. The new strain was dubbed Biotype B, to distinguish it from the more familiar kind (now Biotype A) found throughout Californian vineyards, but which AXR#1-grown vines resist. Biotype B has now been found in six counties.

Overheated comparisons between phylloxera and AIDS, though, are wide of the mark. Rootstocks resistant to Biotype B are known. All that growers need to do is replace their current vines — at a price. "The estimated losses are upwards of \$1 billion for Napa and Sonoma counties, just [because of] Biotype B", says Granett. "Roughly 50,000 acres are at risk in those two counties." The costs are incurred not only in replanting but in the five years of lost production needed for the new vines to become established.

Apart from pulling up the vines and

starting again, there is not, as yet, a practical way of exterminating phylloxera. The best strategy, says Linda Bisson, professor of viticulture and oenology at UC Davis, is one of "peaceful coexistence".

Those with an aggravated sense of the biblical might interpret the advent of Biotype B as just desserts for an industry unwilling to heed advice from (or invest in) UC Davis, the research arm created to foster it. Bisson and Granett may be forgiven for feeling like unwelcome Cas-



sandras, ignored until the disaster they predict finally strikes. "It's highly analogous to an earthquake", says Bisson — scientists point to the signs and bid for research investment, "but until the problem hits, people are unwilling to even listen to you."

Optimists, on the other hand, might see Biotype B as the best thing that could have happened to the industry, in that it will force growers to experiment with new techniques that would otherwise have been uneconomic to attempt. Overall, it will prevent the industry from resting on its collective laurels.

Wine is worth \$3 billion to the state economy, and nine out of ten bottles of wine made in the United States come from California. This could change — promising wineries are springing up all over North America. California could lose out unless its industry continues to match the (proportionately) heavier investment ploughed into wineries elsewhere. □

Vital signs

"WE would like to look at the molecular evolution of the Solar System. This is now the new mission — how life evolved on Earth. Studying the Solar System is the link between the cosmos, and the formation of the elements, and the molecular evolution of life as it formed on Earth." So says Moustafa Chahine, chief scientist of the NASA Jet Propulsion Laboratory (JPL) in Pasadena in the hills above Los Angeles. This visionary view provides the thread that connects JPL's current suite of missions.

JPL has had spectacular successes, most notably with the Voyager missions, but the space race is over, and it has been forced to adopt a leaner image. One small mission, currently under study, typifies the new JPL philosophy — a rapid fly-by of Pluto. The entire science package would have a mass of less than 5 kg, and JPL scientists are working on ways to make ultra-miniature instruments for the purpose. The CCD camera, for example, will tip the scales at less than 200 grams.

Smallness is a part of other projects, too. Each one of a projected series of robot Mars rovers will fit inside a shoe-box. A robot weather station designed for the surface of Mars will have a mass of less than 50 grams.

Such devices represent engineering achievement, but overt science has come to JPL surprisingly recently. In 1978, Chahine, then an engineer working on fluid dynamics, was asked to set up JPL's Science Division, with a task of introducing scientific objectives in on the ground floor of mission design.

Science is in the forefront in JPL's current crop of projects. Magellan has completed the mapping of Venus by radar; Galileo (for all its problems) is on course for Jupiter, and will release a probe into its atmosphere; the Mars Observer (effectively a weather satellite for the red planet) will be JPL's first look at Mars since the Viking missions 17 years ago; the miniature Mars rovers will add the complementary worm's-eye view; and the still-to-be-launched Cassini mission will take another look at Saturn and probe the murky atmosphere of its moon Titan.

JPL is also involved in SETI, the Search for Extraterrestrial Intelligence. Not forgetting the evolving Mission to Planet Earth, which has a more focused objective than it once did, namely to firm up the uncertainties in the global climate system (such as the role of clouds) that are a brake on fuller understanding of global climate change. Taken together, the goal is to establish the physical conditions necessary for life on Earth and elsewhere in the Universe. □

Refining models of nuclear matter

The general expectation that the structure of the dense substance of a neutron star would be surprising has been more than outdone by speculations, but the nuclear data are not yet good enough to tell just what happens.

WHAT exactly is nuclear matter, and where is it to be found? Since the discovery of pulsating stars more than two decades ago, and their identification (by Thomas Gold, see *Nature* **218**, 731; 1968) as stars made primarily from nuclei, electrons and neutrons, the answer to the second question has been taken for granted. But there is a difficulty with the natural view that the interior of a neutron star is simply a collection of neutrons moving about much as if they were inert atoms in a liquid.

If the force holding together a neutron star, believed to be the core of an exploded supernova, is simple gravity, it is difficult to understand how it can be massive enough to hang together against the obvious transition back to hydrogen atoms (for neutrons decay into electrons and protons, on the average in about 20 minutes if admittedly more slowly in strong gravitational fields).

Of course, the core of an exploded supernova contains not just neutrons but nuclei, not least those of the lighter elements involved in stellar nucleosynthesis, so that the interior of a neutron star may be an arrangement of relatively slowly moving nuclei in a sea of neutrons with a uniformly distributed electron gas as well. But then, if the pressure on the core of the star were increased, as it would be if the mass of the star were greater, the nuclei would be disrupted into protons and neutrons. And what if the central pressure were greater still? The constituents of the star would be the quarks of which nucleons are made. What forces would hold matter of that kind together?

What more natural than that the strong nuclear forces are responsible? If that is so, the interior of a neutron star resembles the interior of a nucleus. In other words, the interior of a neutron star is a collection of quarks held together by the gluons that constitute the nuclear cement — or a neutron star is a giant nucleus. That accords with observations (so far as they are complete) of the physical properties of neutron stars.

The density of a neutron star, for example, is more like that of a nucleus than of a molecular liquid. The density of nuclear matter must differ from that of the densest ordinary liquid by a factor of the order of 10^{12} , the ratio of the volume of an atom and of a nucleus. One of the earliest attempts to calculate the properties of nuclear matter in this form (then called "quark matter") was due to George Chapline and Michael Nauenberg (*Nature* **264**, 235; 1976). Starting from the position that the quarks must be

relativistic, they concluded (from General Relativity) that the pressure of the fluid must be roughly a third of the energy-density of the nuclear matter.

But if this is what the interior of a neutron star is like, still more remarkable things are possible. One comes about because quarks themselves are not necessarily stable, but may decay from one into another radioactively by the release of an electron or heavier fermion. And the inverse decays are also possible. So it is reasonable to ask what mixture of quarks and electrons would be the most stable. There is a possibility that nuclear matter made of neutrons may partly consist of electrons, electrically balanced by an array of charged quarks which, if put together into separate nucleons, would make some neutrons and some different number of protons. That is not a surprising possibility, but a likely one. The question is not whether it happens, but under what circumstances, and where.

Even as recently as 1978, it had not been established that the breakdown of neutrons into quarks would happen inside a neutron star. W.F. Fechner and P.C. Joss in that year made a judicious attempt to estimate the likelihood of this phase transition, which is what it would be. Their conclusion was that the existence of stable quark stars was "possible", and that they might well have properties not very different from those inferred from observation and from earlier calculations (*Nature* **274**, 347; 1978).

But now, imaginations have moved further on. For one thing, there is a string of speculative calculations concerned with the effects of nuclear processes within such a star that would liberate neutrinos, then each presumed to have a chance of escaping from the star and carrying away energy. The effect of these processes would thus be to cool a newly formed neutron star with a pulse of neutrinos, perhaps like that observed on the Earth from the Magellanic supernova explosion early in 1987.

The supernova 1987a may be an observational proof of the reality of quark matter, for one of the processes by which neutrinos are emitted consists of the interaction of an *up* quark (one of the three quark components of a neutron) with a negative electron, leaving a *strange* quark and a neutrino. So there is a bias towards the formation of *strange* quarks, whence the term "strange matter" appropriately describing bulk objects consisting predominantly of *strange* quarks. (The term "strangelets" is used for droplets of the stuff.) The possibil-

ity has even been raised that neutron stars of all forms may be metastable against conversion into strange matter.

Strange matter might even have practical value. Not so long ago, G.L. Shaw, M. Shin, R.M. Dalitz and M. Desai suggested making nuggets of strange matter (with mass anywhere between a very small fraction of a gram and many billions of tonnes). Then, they argued (*Nature* **337**, 436; 1989), it would be possible to exploit the reactions by which the quark components of a neutron are converted into strange quarks to generate thermonuclear energy cheaply; just fire slow neutrons at a nugget of strange matter, collect the energy of the neutrinos emitted in some way or another and, in the process, watch the nugget of strange matter grow. A "thermonuclear breeder" is the appropriate term for that.

More recently, people have been brooding about the ways in which ordinary matter would progressively be converted into quark matter or even strange matter. In the form in which nuclei remain intact, the first thing that happens as the density of pressure is increased is that the charge distribution within nuclei is inverted; the energy involved in Coulomb repulsion increases less quickly than the surface energy. Then nuclear matter begins to form, at first as droplets. But then the droplets grow and become rod-like and plate-like ("spaghetti" and "lasagna" are the technical terms).

Earlier this year, C.P. Lorenz, D.G. Ravenhall and J. Pethick from the University of Illinois at Urbana more accurately calculated the equation of state by which the properties of these structures are determined (*Phys. Rev. Lett.* **70**, 379; 1993). Their conclusion is that a smaller proportion than previously thought of the substance of a neutron star is embodied in the outer crust; within that is nuclear matter proper.

H. Heiselberg from the Nils Bohr Institute, together with Pethick and E.F. Staubo from Illinois, have now taken the argument a step further, defining the condition if drops of quark matter are to form within a matrix of nuclear matter (*Phys. Rev. Lett.* **70**, 1355-9; 8 March 1993). They say this picture of the matter inside a neutron star could have important observational consequences; the deformation of the non-spherical oddly shaped drops might be linked to occasional sudden acceleration of the rotation of neutron stars. But, as for the past twenty years, the nuclear data were not good enough to be sure, and are not nearly good enough yet to be sure.

John Maddox

The end of the beginning

Peter Little

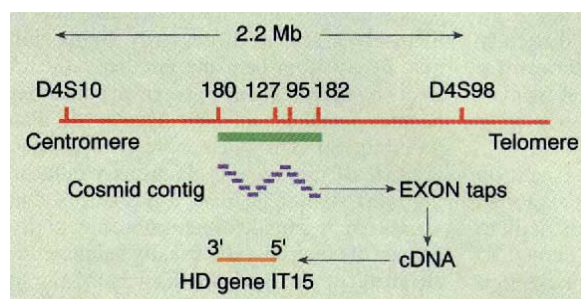
In November 1983, Jim Gusella and a group of 13 co-workers reported one of the first human disease gene-linkage studies¹, showing that a DNA marker, G8, is closely linked on chromosome 4 to the gene that causes Huntington's disease (HD), a very unpleasant, dominant, late-onset, neurodegenerative disorder. An accompanying News and Views² was entitled "The beginning of the end of the dilemma". Now, just under ten years later, a paper in the latest issue of *Cell*³ describes the isolation of the gene that causes HD. What is the gene, and why has it taken a decade of immense labour to identify it?

The intervening period has seen the refinement of restriction fragment length polymorphism analysis, and other polymorphic marker linkage analysis, and the HD gene was pinned down to map position 16.3 on the short arm of chromosome 4: large restriction maps of the region known to contain HD have been generated, many new markers identified and literally thousands of DNA samples from affected families analysed. Cosmid and yeast artificial chromosome (YAC) clones spanning all or parts of the region have been assembled into contigs — this genetic region is one of the most intensively studied segments of the human genome, and the scope of the problem resulted in the setting up of the Huntington's Disease Collaborative Research Group, including Gusella as one of its leading members. It is this group that has published the most complete report of the HD 'gene'.

The figure depicts the 2.2-megabase region of chromosome 4; recombination analysis shows that the markers D4S10 and D4S98 must flank the HD gene. Haplotype analysis (closely linked polymorphisms that are identical between different affected individuals, indicating a shared origin to their disease chromosome) demonstrated that the 500-kilobase region between markers D4S180 and D4S182 should contain the HD gene. This was cloned in a 16-cosmid contig and subjected to intensive 'exon amplification'⁴. This is a powerful technique for identifying coding regions (exons) of genes by using monkey cells (COS-7) to transcribe random DNA fragments inserted in the appropriate vector. If the DNA fragment contains an exon, with its flanking splice sequences, then the cells can splice the transcript: if the DNA does not contain an exon, splicing will not occur. Spliced RNA products can be easily identified by the polymerase chain reaction (PCR) and a

gene fragment, as opposed to a random piece of intergenic DNA, can be isolated from the reaction.

The collaborative group, and others using similar techniques, identified at least three genes in the region. One of these, IT15, is the HD gene. They were able to show this by isolating overlapping complementary DNA clones and sequencing the whole 10,366-base-pair



The Huntington's disease region. D4S10 to D4S98 are marker DNAs: recombination suggests the HD gene lies between D4S10 and D4S98; haplotype analysis suggested that the HD gene should reside between D4S180 and D4S182 (thick green line). Short lines represent cosmid clones and the gene is indicated below. 5' and 3' indicate orientation of transcription, not the actual start and end positions of the gene.

transcript. The 348K protein encoded by the 210-kilobase IT15 gene is unrelated to any existing protein in the databases but at its 5' end, within the putative protein-coding DNA, there are 21 copies of the repeating motif CAG, potentially encoding seven amino acids. PCR-based analysis of this region in unaffected individuals shows that the number of repeats varies between 11 and 34 copies, but in all HD chromosomes the gene contained 42–66 copies — and some even 100 copies. This is the crucial information that enables the group to be confident that it has cloned the HD gene. Trinucleotide repeat expansion is the cause of at least three human genetic diseases (briefly reviewed in ref. 5): myotonic dystrophy, fragile X syndrome and spinal bulbar muscular atrophy. In each case the disease is clearly correlated with expansion of the trinucleotide repeat. The search for the HD gene is apparently at an end.

In view of the immense manpower that has been employed on the search, it is perhaps not surprising that a second group⁶ in *Nature* last week identified a large transcript apparently deriving from the same general region, but using a different observation to conclude that it was a potential HD candidate. Goldberg *et al.* cloned the HD region in three overlapping YACs. They used direct

hybridization selection of cDNAs to isolate seven clones encoded in the region and one of these detected, in two out of 250 HD families, an insertion of DNA into the chromosomal region containing HD. Reasoning that the insertion might be causing disruption of a nearby gene, they searched for this and found a cDNA that detects a 12-kilobase transcript. Based upon this indirect inference, they suggest that their gene is a good candidate for HD. It is not clear that this is the same gene as IT15, although the size might suggest it is, because there is a considerable distance between the location of IT15 and the probe used by Goldberg *et al.* The relationship of the DNA insertion to gene activity is equally unclear. It is a classic illustration of the difficulties of assessing candidacy of a gene, and this gene probably fails on the simplest of tests — that of whether it is mutated in a high proportion of affected individuals. It will not be technically difficult to resolve these questions, in this case, within a short period.

Why has the search for the HD gene been so difficult? At least three reasons can be put forward. Linkage analysis was confounded by three inconsistent patterns in apparent HD individuals, throwing the search area into question. Gene searches were made more difficult because of the large number of genes within the search region, leading to the belief that this is a 'genetic region' — few other regions have been subjected to the technical battering that the HD interval has received, so it is not known if this is actually the case. Either way, a huge amount of labour was required to eliminate candidate genes. The biology of HD was of limited help in selecting target tissues for expression analysis because 60 per cent of all genes seem to be transcribed in neural tissues. The example of the retinoblastoma gene, *Rb*, shows that derangement of expression of a ubiquitously expressed gene can have a tissue-specific pattern of pathology.

Finally, a crucial element of luck only once showed its hand in this search. Unkindly, right at the beginning, it played its part in the choice of a linked marker by random selection of genomic fragments and then deserted all players.

1. Gusella, J. F. *et al. Nature* **306**, 234–238 (1983).

2. Robertson, M. *Nature* **306**, 222 (1983).

3. The Huntington's Disease Collaborative Research Group *Cell* **72**, 971–983 (1993).

4. Buckler, A. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 4005–4009 (1991).

5. Davies, K. E. *Nature* **356**, 15 (1992).

6. Goldberg, Y. P. *et al. Nature* **362**, 370–373 (1993).

Chance has a leading role in these gene searches: a single lucky choice of phage, cosmid or plasmid clone could have changed the outcome dramatically.

The success of the international collaboration is a welcome reminder that science is a collective activity. This group has made a real breakthrough, from which will come reliable antenatal diagnosis using PCR analysis of the trinucleotide repeat. The disease mechanism is still unclear, for it involves a gene of unknown function mutated by trinucleotide expansion of unknown cause and unknown effect. Isolating the gene is, in truth, only the end of the beginning. □

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SOLAR PHYSICS

Seismology makes waves

Jørgen Christensen-Dalsgaard

JUST as earth scientists can investigate the Earth's interior with seismology, so solar physicists can study the Sun's interior. But whereas terrestrial seismic waves are generated by isolated earthquakes (or explosions), solar oscillations arise from persistent gentle and random prodding by turbulence in the outer parts of the solar convection zone. The solar waves are seen as free oscillations of the whole Sun. But on page 430 of this issue¹, Duvall *et al.* show that it is possible, through alternative analyses, to obtain localized information like that obtained from terrestrial data.

In geoseismology, measurements of travel time of waves set up by earthquakes or man-made explosions provide information about the properties along the paths of the waves, from which a three-dimensional picture of the underlying terrestrial structure can be built up. In contrast, the analysis of free-oscillation frequencies in general assumes that the underlying structure has a certain symmetry. Thus, solar frequencies are used to obtain information about the radial variation of quantities such as the sound speed and density, assumed to vary with spherical symmetry. Departure from spherical symmetry can be treated as a perturbation, although axial symmetry is still typically assumed. Thus Goode *et al.*² showed that frequency splitting of solar oscillations can be used to measure latitudinal and radial variations in solar rotation speed.

However, it is patently obvious that the solar surface is far from axisymmetric. An important example is in the dynamics of solar convection: numerical models indicate that convection might be organized in very large scale patterns,

the so-called 'giant cells', yet there is at best marginal indication of such organization on the solar surface. Lavelly and Ritzwoller³ have recently shown how to calculate, under certain simplifying assumptions, the effects of a three-dimensional velocity field on global-mode frequencies. The results are interesting, although the potential effects are too small to be detected at present.

To investigate the properties of local regions of the Sun, local analyses might be preferable. Duvall *et al.* now propose a method for applying an analogue of travel-time analysis to the solar data. The principle of their procedure is straightforward (see Fig. 2). Sound waves propagating in the Sun are refracted owing to the increase of the sound speed with depth; as a result, the ray paths curve back towards the solar surface. The surface phase speed ω/k (ω being the frequency of oscillation and k the horizontal wavenumber on the solar surface) is directly related to the depth of penetration of the ray; hence, by

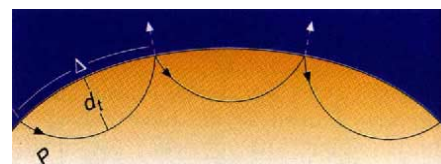


FIG. 2 The propagation of sound waves in the solar interior can be described in terms of acoustic rays. A wave starting at a point P at the solar surface follows a curved path determined by the increase of the sound speed with depth. The depth d_t of maximum penetration is determined by the frequency, the surface horizontal wavenumber k , and the sound speed. At the surface, the wave is reflected. For frequencies exceeding the so-called acoustical cut-off frequency, at approximately 5,000 μHz , reflection ceases to be complete and at somewhat higher frequency the waves are essentially free to propagate into the solar atmosphere. Waves are also attenuated through damping in the immediate subphotospheric layers.

filtering the observed signal according to ω/k , Duvall *et al.* isolate waves penetrating to a given depth. A wave excited impulsively at one point will propagate below the surface, turn up and emerge at a second point, a distance Δ away. Reflected back down, it will re-emerge at a third point and so on, taking the same time t on each traverse. So there should be a strong correlation between the signals at points separated by Δ at sequential times. Attenuation, particularly resulting from imperfect reflection at the surface, gradually weakens the signal, reducing the correlation strength at each step.

In reality, the excitation is probably an ongoing stochastic process, distributed over the solar surface, but it is still the case that points separated in space and time by an integral number of ray lengths and crossing times should be strongly correlated. This is confirmed by Duvall *et al.*, whose analysis was made by correlating a given point with points in circles of increasing radii around it (see their Fig. 1, page 431). The correlation is strong in patches at well-defined separations. The new analysis included modes over a range in penetration depths, so that the separations and correlation times are not precise. But the results still clearly reflect the properties of the rays and this type of analysis allows determination of the local propagation properties of the sound waves.

Duvall *et al.* show that the signal in the time-distance diagram can be improved by averaging over the solar surface. This essentially corresponds to a Fourier transform of the oscillation field; the result is largely equivalent to the often-used $\omega-k$ diagram showing frequency as a function of wavenumber. Although this time-distance diagram really pertains to free oscillations,

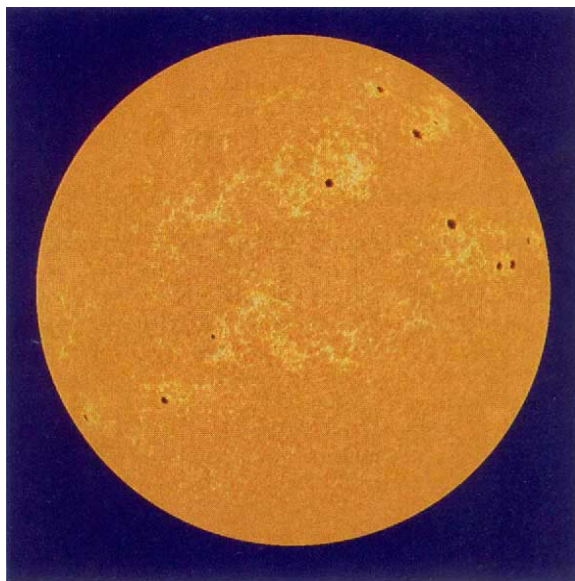


FIG. 1 False-colour image of the Sun at a wavelength of 393 nm, taken from the South Pole. Solar oscillations appear in series of such images as brightness changes.

T. L. Duvall Jr

Duvall *et al.* argue that it may be better for finding sound-speed stratification than the ω - k diagram.

What can we learn from this? Already, Duvall *et al.* have solved a puzzle about the presence of high-frequency solar oscillations. Oscillations persist above 5,000 μ Hz (period around 3 minutes), the expected acoustical cut-off, but is this because of interference effects⁴ or because of anomalously strong reflection at the solar surface⁵? The inversion by Duvall *et al.* shows that the paths of these high-frequency waves make only one return to the surface, which thus seems too diffuse to make a good reflecting boundary for them.

At present, however, the main result is the demonstration that a local analysis is even possible. Evaluation of the diag-

nostic potential of the time-distance diagram probably requires simulations of the response of the observed correlation time and distance to various phenomena beneath the solar surface, such as flows and magnetic fields. But it seems likely that it will develop into a powerful aid to more traditional helioseismic techniques for probing the solar interior. □

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1. Duvall, T. L. Jr, Jefferies, S. M., Harvey, J. W. & Pomerantz, M. A. *Nature* **362**, 430–432 (1993).
2. Goode, P. R., Dziembowski, W. A., Korzennik, S. G. & Rhodes, E. H. *Astrophys. J.* **367**, 649–657 (1991).
3. Lavelly, E. M. & Ritzwoller, M. H. *Astrophys. J.* **403**, 810–832 (1993).
4. Kumar, P. & Lu, E. *Astrophys. J.* **375**, L35–L39 (1991).
5. Balmforth, N. J. & Gough, D. O. *Astrophys. J.* **362**, 256–266 (1990).

MALARIA

That vaccine passes a trial

F. E. G. Cox

ENCOURAGEMENT for those involved in the battle against malaria comes with publication of the results of a large, controlled human phase-III trial in Colombia, in which vaccinated individuals suffered fewer episodes of malaria over a one-year period than people in a placebo group¹. The vaccine concerned has been the centre of contention for some years, as has its architect, the Colombian biochemist Manuel Patarroyo. His persistence now seems to have been vindicated.

Development of a malaria vaccine has become a major challenge, as insecticide and drug resistance have reduced the effectiveness of hitherto successful methods of control. But the task has proved a tough one because the parasite life-cycle involves a series of antigenically complex and diverse stages and the parasites have evolved ways, as yet undetermined, of evading the immune response. The possibilities of a vaccine are based on the observations that most of those infected do develop some degree of immunity, and encouraging results have been obtained in laboratory models. Of the different possible targets, the most favoured is the sporozoite, the infective stage injected by the mosquito vector. But vaccination trials have been equivocal and other targets now include the early liver stages, the blood stages that actually cause the disease, and even the stages in the mosquito taken up after the insect has fed on infected blood².

Approaches to a vaccine have usually involved the systematic application of molecular techniques to identify putative protective antigens, and then to characterize and produce them in a recom-

binant or synthetic form. A completely different tack has been followed by Patarroyo, who empirically synthesized a polymeric vaccine consisting of three blood-stage antigens linked by the dominant element of the sporozoite antigen. This vaccine, known as SPf66, was first tried in the owl monkey, *Aotus trivirgatus*, which is one of the few animal hosts of the human malaria parasite, *Plasmodium falciparum*, and is in plentiful supply in Colombia. The results were encouraging³ and, after a small scale trial on volunteers⁴, Patarroyo embarked on a major human trial⁵ and quickly became embroiled in controversy. This period has yet to be studied by historians, but it was characterized by a combination of envy of Patarroyo and outright disbelief at his results, and genuine scientific criticism. The first trials were indeed not critically controlled and contained no placebo groups, but the latest trial cannot be criticized in this way.

In summary, 739 semi-immune individuals living in an endemic area were given three injections of SPf66 adsorbed onto aluminium hydroxide while 810 received a placebo. Over the next year all the subjects were monitored for malaria: the number of episodes recorded in the vaccinated group was 168 compared with 297 in the placebo group, and the protective efficacy ranged from 22 per cent in 15–44-year-olds to 77 per cent in the 1–4-year-old group, those most at risk.

There are many notable aspects to this study. For instance, 85 per cent of the subjects were of West African origin, indicating that the vaccine would be suitable for Africa where the need for a

childhood vaccine is greatest. From an immunological viewpoint, the most interesting findings are that there was no correlation between antibody levels and protection (meaning that cell-mediated mechanisms may be involved), that continual natural boosting was required to establish strong immunity, and that immunity was specific in that it did not extend to *P. vivax*.

On the face of it, the degree of protection achieved is not dramatic. But it is the first time that such a large trial has been undertaken and there can be no doubt that a certain degree of protection was achieved. This in itself justifies Patarroyo's approach, though it does not mean that the SPf66 is now the only candidate vaccine. It is now a basis for improvement by adding new components to it — as it is safe, and requires some experience of the infection for it to remain effective, one such component might be a transmission-blocking antigen effective only after the infected blood has been taken up by a mosquito⁶, or an anti-disease vaccine⁷, neither of which requires complete protection. In terms of control, mathematical models show that the incidence of malaria would be reduced by a drop in the rate of transmission, which would occur if those infected experienced fewer and less severe episodes of malaria. From the point of view of public health, fewer days would be lost by sickness if everyone were immunized with a vaccine, even only a partially effective one. Malaria is a complex disease, and it may be that all it will be possible to achieve in the near future will be a tolerable level of malaria with which it is possible to live.

SPf66 is being taken seriously by the World Health Organization and other trials are under way. In Ecuador, 193 people have been vaccinated with SPf66 in an area of less intense transmission; although the results were encouraging, the numbers subsequently infected were so few that no firm conclusions can be drawn⁸. The big test will come when, in a joint trial, the Swiss Tropical Institute and the Tanzanian health authorities attempt to find out whether this vaccine protects children in Africa. The trial is now in progress — the results will not be known until July 1994, but they might well determine the future course of development of malaria vaccines. □

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1. Valero, M. V. *et al.* *Lancet* **341**, 705–710 (1993).
2. Cox, F. E. G. *Trends Biotechnol.* **9**, 389–394 (1991).
3. Patarroyo, M. E. *et al.* *Nature* **328**, 629–632 (1987).
4. Patarroyo, M. E. *et al.* *Nature* **332**, 158–161 (1988).
5. Patarroyo, G. *et al.* *Vaccine* **10**, 175–178 (1992).
6. Kaslow, D. C. *et al.* *Nature* **333**, 74–75 (1988).
7. Playfair, J. H. L., Taverne, J., Bate, C. A. W. & de Souza, P. *Immun. Today* **11**, 25–27 (1990).
8. Sempertgui, F. *et al.* *Vaccine* (in the press).

Dorian Gray mice

Robin A. Weiss

NEWLY generated transgenic mice appear to be able to grow indefinitely without ageing, yet with programmed death. This dramatic development is the work of several groups, and has been accomplished in three stages. First, a gene encoding tithonin was introduced from the carp (*Cyprinus carpa*), a freshwater fish that has the capacity for perpetual growth; the *tith* gene confers longevity on mice despite many symptoms of ageing¹. Second, by selecting *tith* mutants with more stable function at 37 °C, the mice show no signs of senescence^{2,3}. Third, by crossing these animals with others expressing mutant *p53* and an inducible *bcl-2* gene, the mice can be triggered to die within 48 hours by apoptosis⁴.

Carp

These new findings bring to fruition ideas pioneered by Sigmund Obispo in the Stoyte Institute of Life Sciences in California. As reported in these columns⁵, Obispo started by inquiring why some long-lived poikilotherm vertebrates such as the carp, and the giant Galápagos tortoise, do not cease growing at sexual maturity, and show little evidence of senescence at the cellular and tissue level. After many a summer investigating various blind alleys, Obispo's laboratory isolated a bacterium, *Campylobacter limnia*, from the jejunum of carp which releases a substance promoting indefinite growth. This growth factor was identified as a small (6K) protein named longevin⁶. Following cloning and expression of the gene for the longevin precursor (preprolongevin), recombinant protein was inoculated into mice. Surprisingly, the treated animals lost their whiskers and developed scales all over the body resembling those on the tails of normal mice⁷. It eventually became apparent that bacterial longevin exerts its effect in fish by forming complexes with a host protein, tithonin, produced by neurosecretory chromaffin cells in the gut⁸. When the tithonin-longevin dimer is administered to adult mice, their life-span is extended unless they develop antibodies to the protein; treatment of young mice delayed the fusion of epiphyses of the long bones.

Tithonin derives its name from Tithonus, the hero upon whom Zeus granted eternal life without eternal youth. In a thought-provoking review⁹, Obispo suggests that by extending normal life-span, he has reversed neoteny, the evolutionary mechanism expounded by Huxley to explain the maintenance of embryonic or fetal features in adults.

Although the *tith* gene is widely conserved among bony fish and reptiles, homologous sequences have not been detected in mice or humans, suggesting that mammals represent natural knockout species¹⁰. To study the phenomenon further, Maunciple *et al.*¹ generated transgenic mice expressing the carp *tith* gene under the control of the pancreatic amylase promoter. Tithonin again inhibited epiphyseal fusion, allowing the mice rapidly to attain a weight of about 300 g before they died of cardiac arrest, at a mean age of 146 days.

Maunciple *et al.*¹ considered that the uncoupling of signal transduction resulting in unlimited growth together with early senescence was due to the lack of longevin in the secreted complexes. But attempts to make longevin transgenics proved to be non-viable, with or without the *tith* gene. It now appears that ageing is due to the extremely short half-life of tithonin in warm-blooded animals.

Two groups have provided evidence that a genetically modified tithonin gene overcomes senescence. Mond *et al.*² used site-specific mutagenesis to achieve a more stable protein. Their transgenic mice grow indefinitely without developing scales, though they become photophobic. Kawaguchi *et al.*³ followed a more elegant route by cloning *tith* from Koi carp that live in the thermal springs on Mount Asama. These fish are so valuable and venerable that it was necessary to employ PCR amplification of *tith* from a single scale to obtain the warm-adapted sequence. The 'K-tith' mice grow to only 1.5 times normal body weight and appear to be healthy in every other respect. It is too early to ascertain their life-span, but none of the heterozygotes has died after more than 600 days. A great deal needs to be done to elucidate the mechanism of *tith*-dependent growth in mammals. Maunciple is looking for a partner protein analogous to longevin. She also has preliminary evidence (personal communication) for activation of *c-myc* in the *tith* mice.

The ramifications of these discoveries are exciting, though potentially disturbing. For example, there is speculation that tithonin might reverse the symptoms of Zachary's progeria (ZP). This is a rare autosomal recessive syndrome with incomplete penetrance, possibly triggered by *Campylobacter* infection¹¹; the disease combines achondroplasia and premature senescence. Although there is no homologue to *tith* in humans, tithonin would be expected to promote bone growth and perhaps delay the ageing process. Retroviral vectors carrying *tith*

complementary DNA are being tested in mice, but research is hampered by the lack of a close animal model for ZP. Another application under investigation is the development of *tith* transgenic cattle. Viandegene Inc. in Chicago are already making progress in this field¹², and the Kobe Soma Corp. are supporting the Japanese team.

One environmental issue is the problem of releasing *tith*-transgenic animals that might never die except by accident or in the abattoir. This is on its way to being solved by linking *tith* expression with apoptosis genes. Ariellos *et al.*⁴ crossed *tith* transgenics with mice constitutively expressing mutant *p53* and conditionally expressing *bcl-2*. Both *bcl-2* and *tith* are linked to a promoter with a response element for the γ -opiate receptor. So long as a minute amount (0.6 ng l⁻¹) of morphine is added to the drinking water, both proteins are synthesized and the animals remain in fine health. On removing the narcotic, nuclei in striated muscle and liver undergo immediate apoptosis. Cell death becomes irreversible within six hours, the body temperature drops and the mice die in 40–48 hours.

Cattle

It comes as no surprise that the generation of ageless, self-destructing higher organisms is viewed with concern. Whereas some welcome the brave new world that has such creatures in it, others predict dire consequences of pursuing this line of research. Alarm was expressed at the recent Genetics Conference¹³, after Bokanovsky suggested that apoptotic death in transgenic cattle would be both humane and would provide tenderized meat. A breeding moratorium was mooted, reminiscent of the Asilomar Conference on DNA cloning 18 years ago. □

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1. Maunciple, V. A., Boone, P. S. & Obispo, S. *Gerontol. Res.* **26**, 958–967 (1989).
2. Mond, M., Marx, B. F. & Watson, H. J. *devl Biol.* **34**, 1044–1056 (1992).
3. Kawaguchi, Y. *et al. Jap. J. expl Endocrinol.* **13**, 1418–1427 (1993).
4. Ariellos, C., Prosperou, K. K., Kaliban, D. W., Mirander, B. P. & Kawaguchi, Y. *Proc. natn. Acad. Sci.* (in the press).
5. Correspondent, A. A. *Nature* **296**, 392–393 (1982).
6. Boone, P. S. *et al. Gerontol. Res.* **8**, 267–271 (1982).
7. Gonister, J. E., Boone, P. S., Maunciple, V. A. & Obispo, S. J. *molec. Dermatol.* **4**, 809–822 (1985).
8. Tennison, A. L. *et al. Growth Form III*, 780–788 (1987).
9. Obispo, S. *Adv. Gerontol.* **6**, 1–39 (1990).
10. Maunciple, V. A. *et al. Am. J. Nutr.* **79**, 2332–2344 (1988).
11. Pfizner, H. O. *et al. Arch. hum. Genet.* **82**, 1413 (1991).
12. Bokanovsky, B. S. & Bokanovsky, S. B. Illinois patent file, no. 2010493A (1992).
13. Cassandra, S. in *Genethics and Transgenics* (ed. Orestes, A. G.) 895–987 (Academia, 1993).

Molecule, assemble thyself!

Edwin C. Constable

CHEMISTS cannot but be impressed by natural molecules' ability to organize themselves into intricate but specific arrangements. The team of Jean-Marie Lehn at Strasbourg University is among the best at mimicking these self-assembling processes, and has now surpassed its previous successes, designing a pair of molecules that spontaneously lock together in small numbers to make a molecular cylinder¹.

Remarkable progress has been achieved in recent years in building unusual and aesthetically pleasing microstructures, whose molecular architectures resemble those of familiar macroscopic objects. The drive for this kind of supramolecular chemistry is the development of functional molecular analogues of mechanical devices². If the correct molecular messages are built into the component molecules of a supramolecular assembly, then simply mixing them together will allow the one to recognize the other and spontaneously form the desired aggregate. Such a process is termed self-assembly, and the understanding of the molecular recognition events involved is one of the urgent targets of supramolecular chemistry.

One of the tricks of self-assembly is to

use a metal ion to control the conformation of a coordinated organic component, and this method has been widely used in the building of helical³, interlinked and knotted⁴ molecular arrays. But such processes commonly involve the controlled interaction of only one type of metal ion with one type of organic ligand. Lehn and colleagues have now extended these processes to self-assembling systems in which a metal ion controls the bringing together of two different organic molecules. The result is a highly complex form of self-organization.

The key component is the molecule hexa-azatriphenylene (compound **1**), which has three sites that may tightly bind a metal ion. The binding of this to a metal ion is driven by the chelate effect which stabilizes complexes in which two or more of the atoms coordinated to a given metal are from the same organic molecule. However, metals are not usually satisfied with only two atoms coordinated to them, and additional species are expected to bind to metal ions attached to the binding sites of **1**. For example, copper(I) (Cu^+) is usually four-coordinate with a tetrahedral distribution of ligands, and so copper(I)

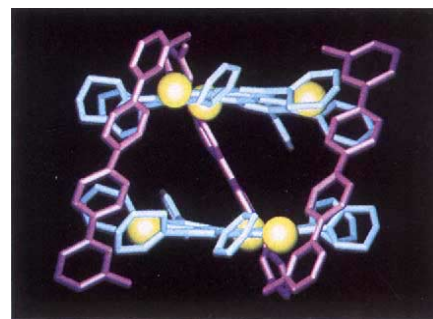


FIG. 2 Side-on view of compound **5**, showing its skewed helical structure.

ions coordinated to **1** would be expected to interact with an additional two donor atoms. Lehn and colleagues demonstrated this by making a second use of the chelate effect by introducing another bidentate ligand, compound **2**.

Sequential reaction of **2** with a copper (I) compound followed by one third as much of **1** allows the spontaneous assembly of the circular complex cation, compound **3** (Fig. 1). This complex may be regarded as a circular cap, with three columnar supports at the circumference. The formation of **3** represents the assembly of an organized structure from seven entities. Lehn then argued that the replacement of the bidentate ligand **2** by the analogue **4**, which can bind to two different metal centres, would lead to the self-assembly of a cylindrical molecule; compound **5**, in which two circular caps are supported by three columns (Figs 1 and 2).

Remarkably, this procedure works, and the interaction of a mixture of **4** and **1** with copper(I) salts gives a deep purple-coloured solution containing the cylindrical hexacation, compound **5**. The group characterized the compound by conventional chemical techniques, and demonstrated that the cylindrical motif persisted in solution.

Lehn's team also determined the X-ray crystal structure of the cylindrical species **5**. This holds one or two surprises. Although the planar caps are coplanar, the columnar supports of the cylinder are not linear and parallel, but are skewed around the cylinder in a triple-helical manner (Fig. 2). This skewing is achieved in two ways; firstly by twisting the individual molecules of **4** about the central carbon-carbon bond that links the two halves of the ligand; and, secondly, by the metal adopting a twisted geometry so that the nitrogen donors on each ligand do not make orthogonal planes.

The cylinder that has been constructed has a central void of height 4 Å and radius 4 Å. Already, the Strasbourg team are working on the inclusion of a suitable chemical prisoner in this cylindrical cage. As Lehn says, the remarkable feature of this molecule is "the

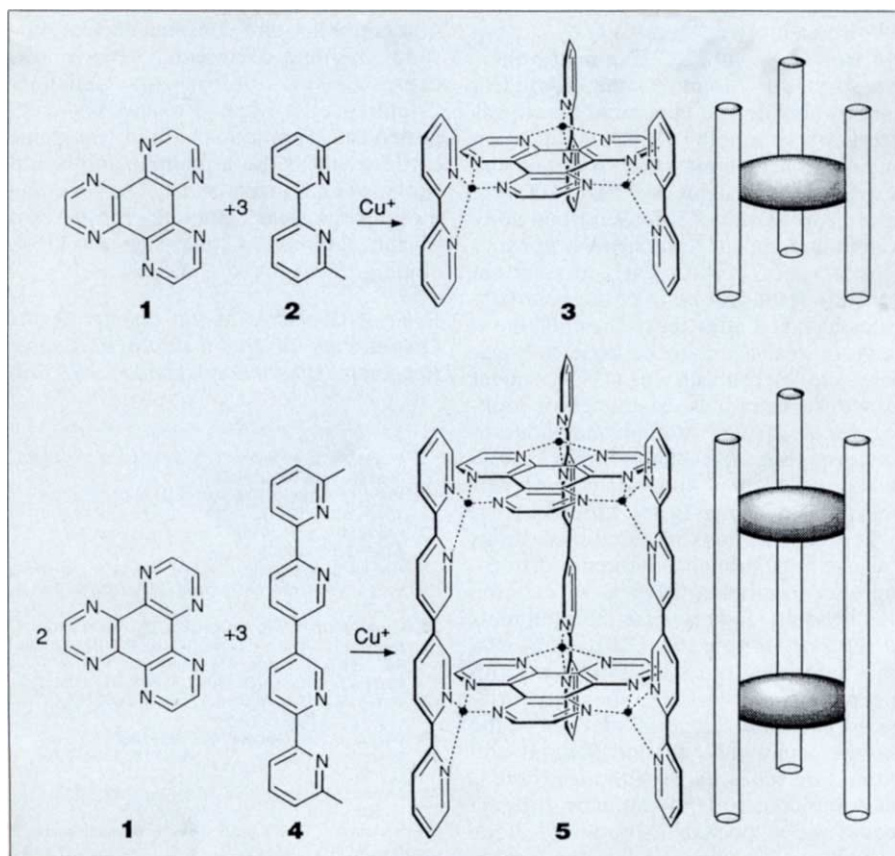


FIG. 1 Reaction schemes for producing Lehn and colleagues' self-assembled cylinders.

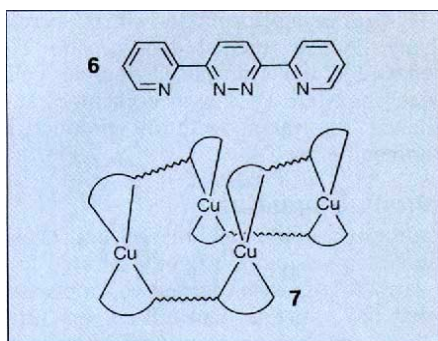


FIG. 3 The ligand **6** used by Osborn and colleagues to make a molecular box, **7**.

spontaneous and correct assembly of altogether eleven particles. . .".

These studies fit into a wider framework of studies involving the metal-directed construction of supramolecular architectures. Whereas Lehn's group has demonstrated the use of a single type of metal to assemble to different ligands, we have demonstrated the ability of one type of ligand to assemble two different metal ions into heteronuclear double-helical arrays⁵. The attractive triple-helical motif of the cylinder **5** has been the target of the group of Williams in Geneva⁶. It turns out that subtle factors control the assembly of double- or triple-helices in a particular metal-ligand system. This observation is borne out by the unexpected helical twisting of **5**.

It is perhaps appropriate to mention a microstructure that has recently been described by the group of Osborn, also in Strasbourg⁷. Compound **6**, which bears an obvious similarity to **4**, reacts with copper(I) to self-assemble a molecular box containing four copper centres, compound **7** (Fig. 3). This compound may be seen as an electron reservoir, and each of the copper(I) centres may be sequentially oxidized to copper(II) without the disruption of the supramolecular architecture. Such compounds provide an entry to new types of microstructures which imitate electronic components. Compound **7** may be thought of as the seed of a molecular accumulator or capacitor which may store and then release up to four electrons to a molecular wire. □

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Ion-channel studies blossom

LEHN's homage to nature is even more obvious in a second paper, describing the synthesis of artificial ion channels that can be incorporated into a lipid membrane resembling a cell wall (M. J. Pregel, L. Jullen & J.-M. Lehn *Angew. Chem. Int. Ed. Engl.* **31**, 1637–1640; 1992). The transport of electrons across membranes constitutes the critical step in photosynthesis, and hence the key step in the production of food and of energy. Similarly,

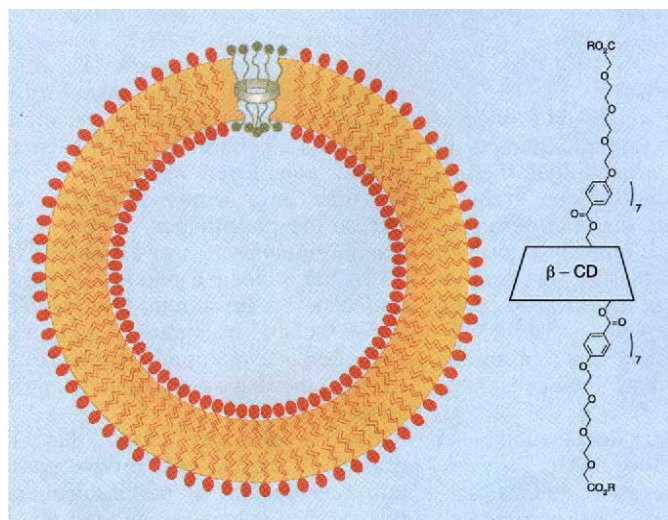


the transmembrane transport of ions is the critical step in metabolism for the controlled production of energy and for the firing of nerve signals. Ions are carried from one side of a membrane to another by one of two processes: by forming complexes with some other agent which can diffuse across the membrane, acting as a shuttle or diffusive carrier; or a large molecule which spans the breadth of the membrane produces a pore through which the ions can easily penetrate.

Lehn and colleagues have made a new class of artificial ion channels by grafting poly(oxyethylene) or polymethylene chains of various lengths onto macrocyclic rings. The authors call their compounds 'bouquet' molecules, as the long chains emanating from the central annulus (with a little imagination) resemble long-stemmed roses protruding from a vase. They have prepared two such molecules, one with a ring-like crown ether as the central ring, the second with a modified cyclodextrin core (resembling a truncated cone).

The authors prepared their lipid vesicles, resembling cells, in lithium chloride solution.

Once these had formed, sodium chloride was added to the solution, so that the composition inside and outside the cells was different. NMR spectroscopy showed that mixing between the two samples was much faster with the bouquet molecules in the vesicle walls. The simplest interpretation is that the metal cations become complexed at one end of the pore, carried through the channel and released at the opposite end.



Top: molecular model of the bouquet molecule based on cyclodextrin (β -CD). Left, structure of the same molecule, showing just two stems. Incorporated into a vesicle wall (far left), the molecule allows rapid interchange of alkali metal atoms between the 'cell' interior and exterior.

Many possibilities can be imagined for using such molecules in studying mechanisms of ion transport and for determining the effect of including other small and large molecules on the effectiveness of cross-membrane channelling. Because these artificial systems are amenable to synthetic manipulation, one could design different lengths of 'bouquets' and choose the best 'flowers' and 'stems' to control selective ion transport to include even non-alkali metals or larger organic cations.

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1. Baxter, P., Lehn, J.-M., DeCian, A. & Fischer, J. *Angew. Chem. Int. Ed. Engl.* **32**, 69–72 (1993).
2. Vögtle, F. *Supramolecular Chemistry* (Wiley, Chichester, 1991).
3. Constable, E. C. *Tetrahedron* **48**, 10013–10059 (1992).
4. Dietrich-Buchecker, C. O. & Sauvage, J.-P. *Bioorganic Chem. Frontiers* **2**, 197–248 (1991).
5. Constable, E. C. & Walker, J. V. *J. chem. Soc., chem. Commun.*, 884–886 (1992).
6. Piguet, C. et al. *J. Am. chem. Soc.* **114**, 7440–7451 (1992).
7. Youinou, M. T., Rahmouni, N., Fischer, J. & Osborn, J. A. *Angew. Chem. Int. Ed. Engl.* **31**, 733–735 (1992).

Better cells for brain repair

Anders Björklund

TAILORING cells for transplantation into the brain is an enticing prospect for improving treatment of certain neurodegenerative diseases. That prospect is behind the work described on pages 450 and 453 of this issue^{1,2}.

At present, replacement of cells in the brain by fetal-cell transplants is being explored as a new therapeutic strategy for such diseases. Immature neurons and glia possess a high propensity for growth and a capacity to integrate structurally and functionally into host brain. Animal experiments have shown that fetal-cell transplants can to some degree substitute functionally for lost neurons in a damaged central nervous system and promote structural repair, and can remyelinate axons in areas subjected to demyelinating lesions. But the wider clinical use of such tissue is problematic, not only from an ethical point of view but also because fetal tissue is heterogeneous and may be difficult to standardize. Moreover, tissue from fragmented fetal material obtained at routine induced abortions may be contaminated, and is potentially in limited supply. The solution to all these problems could lie in generating cells specifically for transplantation. But what kind of cells might be best? Jiao *et al.*¹ and Groves *et al.*² offer two quite different answers — genetically transduced muscle cells (which may seem somewhat surprising) and neural progenitor cells.

Parkinson's disease

The study of Jiao *et al.* should be viewed against the background of attempts to develop a neural transplantation therapy for Parkinson's disease. Here, grafts of fetal mesencephalon, the brain area containing the developing dopamine neurons and neuroblasts, are used to restore dopaminergic neurotransmission in the dopamine-depleted striatum. Clinical trials have given quite promising results and show that fetal-cell transplantation may be feasible in the diseased human brain (as discussed in News and Views³ earlier this year).

At the same time, these studies highlight the problem of obtaining enough viable fetal dopamine neurons for grafting. Dopamine neurons constitute only a few per cent of the fetal mesencephalic cell population, and their survival rate in grafting is likely to be as low as 5–10 per cent. *In vivo* estimates in grafted patients based on positron emission tomography of ¹⁸F-fluorodopa uptake at the transplant site^{4,5} show that the number of surviving cells obtained from 3–4 fetuses is not enough to bring striatal

dopamine synthesis back into the normal range. More complete engraftment is thus likely to require larger numbers of cells spread over wider areas within the striatal complex on both sides of the brain. Fetal neural transplantation thus has a potentially serious drawback: if twice as much tissue is needed to restore normal dopamine synthesis in one striatum, and if bilateral grafting proves necessary, then mesencephalic tissue from as many as 10–15 fetuses may be necessary for fully efficient transplantation in a single patient.

Cells genetically engineered to secrete dopamine may be a way of circumventing these problems. Such cells, which are produced through insertion of the gene encoding the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH), can be highly standardized and produced in unlimited numbers. Moreover, if they could be derived from the patient's own tissues they would carry no risk of immunological rejection. But which types of cells and gene constructs are suitable for intracerebral grafting? Immortalized cell lines, used in the first attempts along these lines, are easy to manipulate genetically but have variable survival rates and are unsuitable because of the risk of tumour formation^{6,7}. The main problem in using primary cells has so far been to obtain high and stable expression of the inserted gene after transplantation.

Muscle cells, as used by Jiao *et al.*, may at a first glance seem like an odd choice. They are neither secretory nor indigenous to the nervous system. But studies on myoblasts that had been genetically engineered to produce growth hormone have shown that such cells release the recombinant protein in substantial amounts *in vitro*, and that they do so for prolonged periods after intramuscular transplantation^{8,9}. Myoblasts can be isolated from muscle biopsies and expanded to large numbers *in vitro*, and so altogether have some quite favourable properties.

Jiao *et al.* now report that TH-transduced muscle cells survive grafting into the striatum of parkinsonian rats, that survival is long-lasting, and that the cells continued to express the TH gene at a stable high level for the full duration of the six-month investigation. A functional level of dopamine secretion, as assessed by a motor asymmetry test, was apparently also maintained. This is a clear improvement over previous studies using transduced fibroblasts¹⁰, where the expression of the inserted TH gene declined over time, and the functional effects were transient. In contrast to the

TH-expressing fibroblasts, which secrete only DOPA (the product of the TH enzyme), the transduced muscle cells decarboxylate DOPA to dopamine, and hence the main secretory product is dopamine itself.

Crucial question

Although stable cell survival and transgene expression is a significant step forward, it is most important to emphasize that the available data tell us very little about the *in vivo* functional efficacy of the TH-transduced muscle cells in the rat model for Parkinson's disease. Drug-induced rotation, which is the functional test used by Jiao *et al.*, gives a helpful measure of the level of tonic dopamine secretion from the transplanted cells, but it does not provide any information on their ability to ameliorate any of the neurological deficits seen in tests of spontaneous (that is, non-drug-induced) sensorimotor behaviours. This is a crucial question that will have to be explored in detail in the rat and primate Parkinson models. It remains to be shown whether cells of a non-neuronal nature which secrete dopamine in a non-regulated constitutive manner can exert any such effects.

The strategy pursued by Groves *et al.*², based on isolation and *in vitro* proliferation of progenitor cells derived from the central nervous system itself, is a particularly intriguing alternative way of obtaining cells for intracerebral grafting. Noble and collaborators have previously shown that oligodendrocyte precursors can be maintained in an undifferentiated, continuously dividing state in the presence of a combination of platelet-derived growth factor and basic fibroblast growth factor. As was the case in the myoblast experiment, this allowed Groves *et al.* to expand the oligodendrocyte precursors to high purity. When transplanted into a demyelinated region of the spinal cord of adult syngeneic rats, the vast majority of the axons throughout the demyelinated area — in some cases more than 90 per cent — were remyelinated within three weeks.

It remains to be seen whether this

1. Jiao, S., Gurevich, V. & Wolff, J. A. *Nature* **362**, 450–453 (1993).
2. Groves, A. K., Barrett, S. C., Franklin, R. J. M., Crang, A. J. & Mayer, M. *Nature* **362**, 453–455 (1993).
3. Gage, F. H. *Nature* **361**, 405–406 (1993).
4. Sawle, G. V. *et al.* *Ann. Neurol.* **31**, 166–173 (1992).
5. Widner, H. *et al.* *New Engl. J. Med.* **327**, 1556–1563 (1992).
6. Wolff, J. A. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **86**, 9011–9014 (1989).
7. Horellou, P., Marlier, L., Privat, A. & Mallet, J. *Eur. J. Neurosci.* **2**, 116–119 (1990).
8. Barr, E. & Leider, J. M. *Science* **254**, 1507–1509 (1991).
9. Dhawan, J. *et al.* *Science* **254**, 1509–1512 (1991).
10. Fisher, L. J., Jinnah, H. A., Kale, L. C., Higgins, G. A. & Gage, F. H. *Neuron* **6**, 371–380 (1991).
11. Renfranz, P. J., Cunningham, M. G. & McKay, R. D. G. *Cell* **66**, 713–729 (1991).
12. Snyder, E. Y. *et al.* *Cell* **68**, 1–20 (1991).
13. Reynolds, B. A. & Weiss, S. *Science* **255**, 1707–1710 (1992).

level of myelin repair is stable over longer post-operative times and is associated with significant functional recovery. Nevertheless, the results indicate that the progenitor cell strategy may have considerable potential for repair of demyelinating lesions in the central nervous system. Although Groves *et al.* used a syngeneic graft-host combination, previous experience with allogeneic cell suspension transplants indicates that cells from a genetically dissimilar donor may be equally effective.

The progenitor cell strategy is, however, likely to have wider implications for intracerebral transplantation. It may be possible¹¹⁻¹³ to generate from the central nervous system progenitor cells that can differentiate not only into oligodendrocytes but also into cells with

neuronal and astrocytic properties. Such cells may very well turn out to be ideal gene carriers or vehicles for gene transfer. Not least, the ability of the neuronal and glial progenitors to migrate from the site of implantation and integrate structurally with the surrounding host tissue may be particularly favourable in gene-transfer experiments, especially in the Parkinson model. Precursor cell biology and gene-transfer technology is therefore likely to play a large part in the future development of rational cell-transplantation strategies in neurological diseases. □

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SOLAR SYSTEM

Dust from outer space

Joseph A. Burns

GRAINS from interstellar space, from beyond our Solar System, have been discovered for the first time, by a sensitive dust detector aboard the Ulysses spacecraft. This result, reported by Grün *et al.* on page 428 of this issue¹, reaffirms a truism of astronomical research: that the unexpected will be discovered whenever instruments are significantly improved or new regions of space are explored². To underline the point, Grün *et al.* report here and elsewhere³ three other important results from Ulysses' flyby of Jupiter 13 months ago. Contrary to previous evidence, remarkably little material was found within Jupiter's gravitational grasp; at greater distance (well beyond Jove's gravitational sphere of influence) submicrometre grains were found streaming away from the massive planet; and these latter particles arrived at the spacecraft only during periodic bursts.

Interplanetary dust is of interest because it is thought to be primitive material, either the end-point of innumerable asteroid collisions and/or debris spewed from short-period comets⁴. Although individual particles are generally tiny and widely separated in space, the dust complex as a whole that envelops the inner Solar System is visible from ground as the faint glow of zodiacal light; single grains have — after being gently decelerated by the Earth's atmosphere from their interplanetary

trajectories — been captured by high-flying aircraft to be returned for laboratory scrutiny. In addition, space vehicles have borne dust detectors of various designs in order to determine the properties of interplanetary grains in the region of the terrestrial planets.

However, until the Ulysses flight, little

detector is much more sensitive, identifying grains that are, depending on speed, greater than only several hundredths or tenths of a micrometre; in addition, this sophisticated device can determine the electrical charges, speeds and flight directions of all measured grains.

Although dust in the inner Solar System generally orbits the Sun on prograde, slightly elliptic and inclined paths like its putative asteroid and comet sources, in Jupiter's locale Ulysses found most so-called 'big grains' (those larger than about half a micrometre in radius) to be moving along retrograde paths, the opposite way around the Sun to the planets. As the grains are observed to have speeds well above that necessary to escape the Solar System and as they are streaming in from the direction that interstellar helium flows past the Solar System, Grün *et al.* identify them as interstellar grains. This is the first direct detection of interstellar material, although various meteorite inclusions have been ascribed to interstellar sources. One must wonder how the particles, which — as they should have low densities — feel a repulsive solar radiation pressure larger than the Sun's gravity⁶, can penetrate so deeply into our Solar System. This discovery of interstellar material opens up the tantalizing possibility that interplanetary probes can be used to sample the isotope and chemical stew of interstellar space.

A second surprise from the Ulysses instrument is that a mere nine impacts were recorded³ within 50 jovian radii of the planet (one radius = 71,398 kilometres); six of the grains seem to be interplanetary interlopers that are gravitationally focused by the weighty planet. This scarcity of bound material in the realm of the galilean satellites presumably reflects the difficulty of getting impact ejecta off Jupiter's large moons. A puzzle is that, despite their much cruder detectors, the Pioneers had measured fifteen impacts in roughly this same region.

Perhaps the explanation is simply that the earlier spacecraft had plunged close to Jupiter and had skimmed the dust-laden ring system^{5,7}.

Another unforeseen discovery is that, starting 60 days before Jupiter was reached, the number of quite small (0.1 micrometre) particles increased¹; such grains continued to be over-abundant for several months after encounter. These dust specks were moving well above the Solar System's escape speed and were

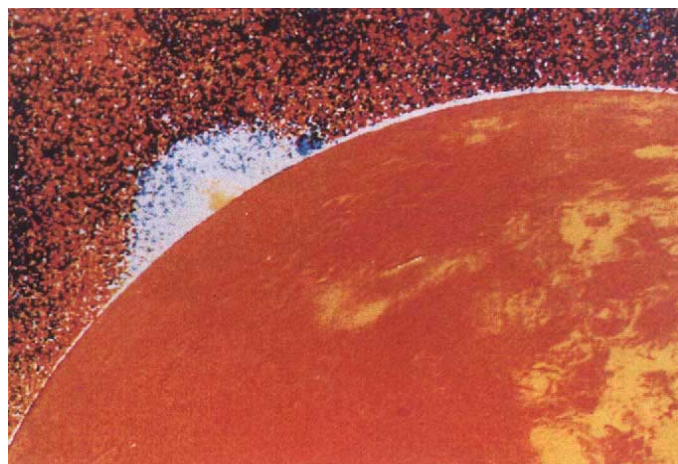


FIG. 1 Volcano on Jupiter's innermost galilean satellite, Io — a source of jovian dust detected by Ulysses? (Computer-enhanced, false-colour image from Voyager 1.)

was known about the small particle population beyond Mars' orbit. The spectacularly successful Voyager mission allotted no instrumentation for sensing interplanetary dust. The only measurements in the vicinity of Jupiter were made in the late 1970s by Pioneers 10 and 11, which carried pressurized "beer-can" detectors that could be punctured by hypervelocity projectiles larger than about 10 and 20 micrometres, respectively⁵. By contrast, the refined Ulysses

received only when the detector pointed towards the planet. Clearly these grains are being ejected from the Jupiter system: yet it is surely counter-intuitive that any material should escape from the deep gravitational well of the most massive planet.

The key to this unexpected motion may be that, because matter in space is charged, electromagnetic forces can alter

above 10 kilometres a second. According to this mechanism, any source of dust in the jovian system (such as Jupiter's gossamer ring as well perhaps as Io's volcanoes; Fig. 1), if beyond synchronous orbit at 2.25 jovian radii, might be a supplier for the speeding small grains that Ulysses detected in interplanetary space. It is interesting to speculate on the applicability of this process to the other giant planets.

ESA/Astronomy Now

Almost all the small grains ejected from Jupiter have been discovered to arrive at Ulysses in narrowly collimated streams of short duration spaced at intervals of 28 ± 3 days; six clumps, two before closest approach and four after, were observed. What could impose such regularity? It is unlikely to be produced at Jupiter because any clustering there would be washed out in the long flight to Ulysses. A credible cause of the periodicity in the bursts is the solar wind's convected magnetic field, which can reverse sign with a period close to that of the Sun's coronal rotation (26–29 days). As Morfill and Grün⁹ have pointed out, the motions of submicrometre grains in

space are dominated by Lorentz forces due to the interplanetary electromagnetic field. But the details of this interaction remain to be worked out.

A similar dust detector is aboard the ill-fated Galileo mission and will start orbiting Jupiter in December 1995. An improved version is scheduled to be carried by the Cassini spacecraft when it starts looping about Saturn a decade hence. In the meantime Ulysses, having swung by Jupiter, is now returning to the inner Solar System, to give us our first ever views of the Sun's poles. If the truism still holds, disappointment will not be an option. □

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1. Grün, E. *et al.* *Nature* **362**, 428–430 (1993).
2. Harwit, M. *Cosmic Discovery* (Basic Books, New York, 1981).
3. Grün, E. *et al.* *Science* **257**, 1550–1552 (1992).
4. McDonnell, J. A. M. (ed.) *Cosmic Dust* (John Wiley, New York, 1978).
5. Elliot, J. & Kerr, R. *Rings* (MIT Press, 1984).
6. Burns, J. A., Lamy, P. L. & Soter, S. *Icarus* **40**, 1–48 (1979).
7. Showalter, M. R., Burns, J. A., Cuzzi, J. N. & Pollack, J. B. *Nature* **316**, 526–528 (1985).
8. Burns, J. A. in *Origin & Evolution of Interplanetary Dust* (eds Levasseur-Regard, A. C. & Hasegawa, H.) 341–348 (1991).
9. Morfill, G. E. & Grün, E. *Planet. Space Sci.* **27**, 1283–1292 (1979).

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

FIG. 2 Ulysses in 1989, now boldly going where no probe has gone before.

the dynamics of any particles carrying large charges. Whereas uncharged bodies will move along keplerian ellipses, the most highly charged particles (or the smallest ones for a given surface potential) will spiral about the local magnetic field lines, much like the constituents of a magnetospheric plasma. Bodies carrying intermediate charges may have quite complex motions, in which orbits evolve⁸; for the expected electrical potentials of a few volts, bodies in the 0.01–0.1 micrometre range fall into this class.

Thus to understand grain trajectories, one must know Jupiter's electromagnetic environment. Close in to the planet, the ambient magnetic field is, crudely, a tilted dipole. In addition, as a consequence of the rotating magnetosphere, there is a radial electric field. Positively charged grains will be accelerated outwards along this field. Although previous estimates were that circumjovian dust would attain negative potentials, M. Horanyi (University of Colorado) has recently argued that, for certain plasma parameters, grains in Jupiter's inner magnetosphere may be positively charged. Some of these particles, those with specific charge-to-mass ratios, will be repulsed by the planet and will reach interplanetary space with speeds well

DAEDALUS

Floating goats

Being a research animal, says Daedalus, is a highly skilled career these days. Starting from the simple Skinner box with its one lever, researchers have moved on to such complex masterpieces as computer-controlled vending and games machines for chimpanzees, and systems by which cats and mice can control their own lighting. DREADCO biologists are in the forefront of such research. They are finding out, for example, if a cat can learn to drive a simple feline car. If it can, will it thereafter decline to walk? Will a rat see any benefit in squeaking louder through a microphone? Can an octopus operate a molluscan video machine? And can a goat learn to navigate a river?

This last question is strictly practical. Many subtropical lakes and rivers are increasingly choked by floating plants like the water hyacinth. Marshes and waterways in more temperate lands are colonized by impenetrable reeds. If melting polar ice raises the world's water levels, more and more land will be submerged, and taken over by such intractable aquatic plants.

Hence Daedalus's floating goat. On land, the goat is renowned for its cursed enterprise and intelligence, and its ability to eat almost anything. It is practically an incinerator on legs. Farmers use goats to clear brushy plants from open country; indeed, they are blamed for reducing much of Africa and the Middle East to the present desert. A herd of waterborne goats should have a devastating impact on the vegetation choking a lake, a waterway or a marsh.

DREADCO engineers are devising a sort of autoinflating life-jacket to fit round a goat. The animal should rapidly get used to this novel form of harness. When it enters water, the life-jacket will inflate, and flipper extensions will project down to be moved by the creature's legs. Like most creatures, a goat swims by continuing its normal walking leg motion. It will find to its astonishment that it floats stably, with its head well out of the water, and (thanks to the flippers) can move at speed. Soon it will be paddling about vigorously, and manoeuvring like a boat. It will be able to browse on water plants far beyond its normal reach. When it returns to shore, the life-jacket will deflate and revert to an inoffensive harness.

Waterborne goats will clear huge amounts of plant-choked wetland, freeing it for fishing and human navigation. A slow leak in their life-jackets will teach them to return at intervals to their home farm, thus keeping them domesticated. From the useless reeds and water plants, they will produce valuable milk, cheese, wool, flesh and leather.

David Jones

What makes popcorn pop

SIR — Corn and popcorn belong to the same plant species *Zea mays mays*¹, but only popcorn pops. Previous studies on this question have been restricted to popcorn fruits and have demonstrated that pericarp (hull) and starch are important for popping^{2,3}.

We compared four distinct varieties of corn, C10, C12, C16 and C08, and three popcorn varieties, P60, P61 and P62. We measured thermal diffusivity of the pericarp using a photoacoustic technique⁴. Popping values, the ratios of expanded to original volumes, were 28.3, 32.2 and 33.5 in P62, P61 and P60, respectively. In the corn varieties, popping values were 1.7, 3.9, 4.4 and 8.7, respectively, for C12, C08, C10 and C16. These results indicate a clear difference in popping ability between popcorn and corn types. We found highly negative simple Pearson correlations between expansion capacity and kernel weight ($r=-0.91$), kernel volume ($r=-0.91$) and proportion of opaque/brilliant endosperm ($r=-0.77$). We also found a highly positive correlation between expansion capacity and pericarp thickness ($r=0.81$), and positive correlations between the proportion of opaque/brilliant endosperm and kernel weight ($r=0.88$) and volume ($r=0.89$). These findings indicate the importance of both the pericarp thickness and the endosperm starch

type to popping quality.

Popcorn pericarp has a thermal diffusivity and a thermal conductivity on average 2.9 and 2.0 times higher, respectively, than those of the corn pericarp. We found a high positive correlation between the thermal diffusivity and the expansion capacity ($r=0.94$). The higher values of the thermal diffusivity and conductivity suggest that the popcorn pericarp is less amorphous, that is, more structurally organized, than the corn pericarp.

Birefringence measurements of the pericarp (expressed as optical path difference) gave an average value of 56.7 nm for popcorn and 38.1 nm for corn pericarp ($t=7.42$, that is, significantly different at the 0.1% level) in the less birefringent sectors (brilliant). The most birefringent areas displayed values of 93.6 nm for popcorn and 64.8 nm for corn pericarp ($t=12.81$), indicating a more crystalline arrangement of cellulose and a higher degree of fibrillar packing in popcorn pericarp compared with corn.

We carried out popping and puffing experiments to investigate the contribution of pericarp and type of endosperm starch to popping characteristics (see figure). Pericarp extraction in popcorn varieties reduced popping by 90.4%, confirming earlier studies². In hulled kernels, popcorn starch was 62.2% more suitable for popping than was that of corn endosperm. Thus the pericarp characteristics, rather than those of the endosperm, seem to be most important for popping.

The puffing experiment showed that the starch quality of corn kernels also has the potential to produce good expansion ability, for in this case, the metal cap of the pressure chamber replaced the weak corn pericarp. Assuming a pressure vessel model^{2,5,6} for the popping mechanism, where the pericarp acts as a vessel cap, we can draw two conclusions. First, using our thermal data, the popcorn pericarp heat-transfer coefficient is roughly 1.9 times that of corn. This implies a more efficient heat transfer into the popcorn kernel, so that its inner temperature grows faster and higher than that of corn pericarp, while the endosperm temperature stays lower. The lower temperature of the popcorn pericarp minimizes the chances of its combustion when the starch granules are at a temperature producing maximum expansion. On the other hand, the corn pericarp becomes dark and calcined at the temperature of maximum starch expansion. Second, the higher mechanical resistance of popcorn pericarp (approximately four times that of corn pericarp)

means that it can sustain higher pressure, favouring high popping in popcorn varieties. The thermal and mechanical properties of the pericarp show that the pericarp acts as a cap.

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1. Doebley, J. F. & Iltis, H. H. *Am. J. Bot.* **67**, 982–993 (1980).

2. Hoseney, R. C., Zelesnak, K. & Abdelrahman, A. J. *Cereal Sci.* **1**, 43–52 (1983).

3. Haugh, C. G., Lien, R. M., Hanes, R. E. & Ashman, R. B. *Trans. Am. Soc. Agric. Engng* **176**, 168–171 (1976).

4. Vargas, H. & Miranda, L. C. M. *Phys. Rep.* **161**, 43–101 (1988).

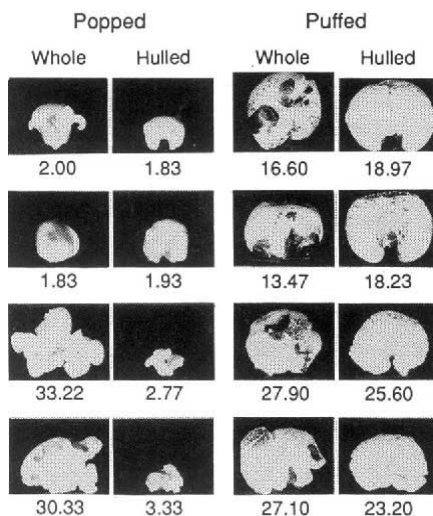
5. Strehlow, R. A. *Combustion Fundamentals* (McGraw-Hill, New York, 1984).

6. Semenov, N. N. *Chemical Kinetics and Chain Reactions* (Oxford Univ. Press, 1935).

Sperm costs and lifespan

SIR — Van Voorhies reported in his study on the nematode *Caenorhabditis elegans*¹ that sex reduces the lifespan of males, but not of hermaphrodites. Because hermaphrodites do not produce any sperm during mating (in contrast to males), he assumed that spermatogenesis is the constraining factor in reproduction. To test this, he used mutants with deficient spermatogenesis. With them, he claims to have shown that the production of sperm rather than the physical act of mating has a significant impact on male lifespan.

If this were true, one would also expect to find an elongated lifespan of unmated mutant males compared to unmated wild-type males (which produce more sperm). However, the data presented in Figs 2 and 4 of Van Voorhies's paper suggest that there is no difference in the lifespan of unmated wild-type (Fig. 2) and unmated mutant males (Fig. 4). Even if these two figures represent two different sets of experiments, the conditions under which they were done



Kernel expansion in (from top row to bottom row) two varieties of corn, C10 and C12, and two of popcorn, P61 and P62, subjected to popping and puffing with whole and hulled kernels. Numbers indicate pop expansion, the ratio of the expanded to the original volume. For the popping process, a sample of 30 g of seeds was put in corn oil at a temperature of around 180 °C. Puffing was done by placing a 160-g sample of kernels in a rotatory pressure chamber (Canon) heated by a propane flame at 180 °C with an internal pressure of 135 p.s.i.

must have been virtually the same as the lines of wild-type mated males are very similar in the two graphs. Therefore, we believe that Van Voorhies's conclusions concerning the significance of spermatogenesis in *C. elegans* are not yet justified. There may be other important factors involving costs before or during mating that have influenced his results (for example any unknown effects of the locus *spe-26*).

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VAN VOORHIES REPLIES — Frischknecht and Wedekind raise two major points about my paper¹. First, if spermatogenesis is the main factor affecting *C. elegans* lifespan then male worms with mutations blocking spermatogenesis should live longer than wild-type worms. I showed a large increase in lifespan of mutant hermaphrodites, but no such increase in male lifespan. I agree that this implies that there may be other factors affecting lifespan and that the *spe-26* mutations might have pleiotropic effects in addition to blocking spermatogenesis, and I am now doing experiments to test these possibilities.

Frischknecht and Wedekind's second point, that mating *per se* could reduce lifespan, is excluded by my data: the mutant males apparently engage in all normal mating activities, yet their lifespan is slightly longer than unmated males (my Fig. 4). Additional support for the assertion that the act of mating or premating activity are not the factors reducing mated male lifespan comes from an experiment in which wild-type males were mated only for 30 hours and then isolated from hermaphrodites. Survivorship in this group of males was similar to that of males kept with hermaphrodites throughout their entire lives.

This contrasts sharply with results in *Drosophila*, where the activity of mating reduces lifespan. Male *Drosophila* mated for a short time and then removed from mates assume the survivorship pattern of flies which have been unmated their entire lives².

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2. Partridge, L. & Andrews, R. J. *Insect Physiol.* **31**, 393–395 (1985).

Aluminium in Alzheimer's?

SIR — Landsberg *et al.*¹ reported the absence of evidence for aluminium in senile plaque cores of cases of Alzheimer's disease. They used particle-induced X-ray emission (PIXE) for elemental analysis of brain tissue sections immunostained for complement factor C3d, and of unstained sections. The authors concluded by questioning the potential role of aluminium in the aetiology of Alzheimer's disease. But their studies focused exclusively on senile plaques in an attempt to replicate the findings of Candy *et al.*² of high concentrations of aluminium and silicon in colocalization with this structure.

It is important to note that the evidence linking aluminium to Alzheimer's disease rests primarily on the association of this element with neurofibrillary tangles rather than senile plaques. Indeed, data showing aluminium accumulation in neurofibrillary tangle-bearing neurons has been obtained from Alzheimer's disease brains^{3,4} and from brains with Guam ALS/parkinsonism-dementia complex using scanning electron microscopy with energy-dispersive⁵ and wavelength-dispersive X-ray analysis⁶, laser microprobe mass analysis (LAMMA)⁷, secondary ion mass spectrometry (SIMS)⁸ and histochemistry⁹. In these studies, both stained^{4,5} and unstained^{4,6} specimens have yielded positive results. The consistent finding of aluminium in association with the neurofibrillary tangle, using such widely differing analytical approaches, suggests that chance contamination cannot explain these results.

We agree that it is important in microanalytic studies of this type carefully to control for various sources of exogenous contamination. However, using LAMMA, where tissues are viewed and analysed in semithin plastic-embedded sections^{5,10}, non-biological particles can be identified and avoided. As Landsberg *et al.* noted, in 1986 we reported a failure to identify aluminium or silicon accumulation in senile plaque cores of Alzheimer's disease¹¹ using LAMMA with an elemental detection limit in the one p.p.m. range. Finally, analysis of unfixed, unstained cryostat sections of affected brain tissues has yielded comparably prominent aluminium-related peaks within neurons in tangle-rich areas^{4,6}.

Together, these data strongly suggest that the association of aluminium with the neurofibrillary tangle represents a biological characteristic of this key pathological feature of the disease rather than a postmortem artefact. The large

body of positive scientific evidence of aluminium accumulation in neurofibrillary tangles has not been directly questioned by Landsberg *et al.*¹. This evidence remains the basis by which this potentially toxic element could be linked to Alzheimer's disease.

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1. Landsberg, J. P. *et al.* *Nature* **360**, 65–68 (1992).
2. Candy, J. M. *et al.* *Lancet* **i**, 354–357 (1986).
3. Perl, D. P. & Brody, A. R. *Science* **208**, 297–300 (1980).
4. Good, P. F., Perl, D. P., Bierer, L. M. & Schmeidler, J. *Ann. Neurol.* **31**, 286–292 (1992).
5. Perl, D. P. *et al.* *Science* **217**, 1053–1055 (1982).
6. Garruto, R. M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 1875–1879 (1984).
7. Perl, D. P. *et al.* *J. Neuropath. exp. Neurol.* **45**, 379 (1986).
8. Linton, R. W. *et al.* *Trace Elements Med.* **4**, 99–104 (1987).
9. Picardo, P. *et al.* *Acta Neuropath.* **77**, 1–4 (1988).
10. Perl, D. P. *et al.* in *Basic and Therapeutic Strategies in Alzheimer's Disease* (eds Fisher, A., Hanin, I. & Lachman, C.) 241–248 (Plenum, New York, 1986).
11. Stern, A. J. *et al.* *J. Neuropath. exp. Neurol.* **45**, 361 (1986).

Maker's mark

SIR — We would like to present evidence for the existence of a controlling influence in the construction of human chromosome 4, and possibly others.

Searching the GENBANK/EMBL combined DNA sequence database (release 74) with a short piece of the cloning vector pBSISK+ reveals a puzzling number of near-perfect matches to sequence tagged sites (STS) on human chromosome 4. For example, the first 50 bases of HUM4STS184 show perfect homology to the multiple cloning site of pBSISK, as do sequences in HUM4STS182, HUM4STS170, HUM4STS183, HUM4STS181, HUM4STS311 and HUMXT009966. We can only interpret this to mean that man, unlike most other species, was indeed 'manufactured', presumably by some all-powerful deity. This individual must have used standard cloning techniques to piece together chromosome 4 (did he/she use different vectors for different chromosomes?). Despite leaving behind evidence of the construction, this work is clearly a *tour de force*.

The company that sells pBSISK+ must, presumably, have a licence from God to market and sell his vector. One wonders, perhaps, if other well-known repetitive elements, for example Alu sequences, represent the fossil remains of earlier divine cloning vectors.

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Eye, brain and vision

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Vision and Visual Dysfunction. General editor: John Cronly-Dillon. *Macmillan*: 1991. 17 volumes. Approximately 5,000 pages. £1,250, \$2,295.

VISION and Visual Dysfunction is no lightweight: indeed its seventeen volumes weigh in at nearly 42 pounds. Fifty years ago, everything worth knowing about vision could have been comfortably packed into a single volume: one wonders what has happened since to justify the size of this work. We have, it is true, come close to understanding certain phenomena, such as colour vision and stereopsis, though in both cases there are annoying facts at the fringes for which there is still no agreed theoretical explanation. Motion perception is also less of a mystery than it once was, but here the areas of doubt are wider. There has been an explosion of work on the neurophysiological basis for the performance of such visual tasks and in all three cases its results tally reasonably well with the psychological findings. Many other areas are less well understood: despite David Marr's valiant effort, we are still only groping our way towards an understanding of object recognition. Except in the case of early visual processing (where Marr's ideas followed neurophysiological findings), neurophysiology has rarely led the way towards an explanation of how such tasks are achieved: the basic mechanisms of colour vision were discovered some time before colour opponent cells were identified.

Despite the march of progress, there are some topics on which little or no advance has occurred over the past 50 years. The constancy of shape and size is one example. Moreover, we have no understanding of how certain very simple visual tasks are performed. If an observer is presented with a square and a circle horizontally aligned, regardless of the parts of the retina to which they project, he can detect rapidly and accurately which is to the left (or right). This phenomenon is an example of 'transposition', a term that does not appear in this book's index. Another easy task is seeing that one thing is inside another, whether in two dimensions or three; again we have no idea how this is done.

Such tasks can be readily performed by computer, but unfortunately not by methods that have much plausibility, given what we know about the nervous system. Nevertheless, artificial intelligence has helped our understanding of some problems, for example the estimation of depth from shading or the use of vertical disparities in stereopsis. It can

help to define the nature of a task and can suggest possible mechanisms by which it is performed by the visual system.

The awesome array of facts gathered over the past 50 years and presented in this work owes something to the development of new techniques. The neurophysiologists invented the micro-electrode and have greatly refined its use. Not to be outdone, psychologists have developed increasingly ingenious techniques for elucidating the properties of the visual system. They include random dot stereograms, random dot kinematograms and the cancellation method of investigating opponent colour processes. Moreover, psychophysical data can now be analysed by much more sophisticated methods, including the use of signal detection theory.

It is, however, to be feared that the tendency to jump on bandwagons has sometimes led to the publication of more and more papers at the expense of discovering less and less. The craze for the specification of stimuli in Fourier terms may be useful as a descriptive device, but has failed to produce any revolutionary theoretical insights. Does it really matter how many spatial frequency channels there are or whether they are continuously distributed?

Another recent fashion, particularly in Europe, is neuropsychology — the investigation of the effects of brain damage on vision. The results are bedevilled by the fact that no two patients have the same lesion: indeed perhaps no two have their cortices organized in the same way outside the primary areas. It is as though one were trying to solve a jigsaw puzzle in which both the shape of the pieces and the patterns on them are constantly changing. The observations made in such studies have a certain bizarre interest, but have thrown little light on the organization of the visual system. We need to know not where but how a visual task is performed. It is difficult to think of any visual disturbance that cannot result from a brain lesion. There is a story that the late Oliver Zangwill, on being presented with an agnostic patient, began the first interview by displaying his fountain pen, while asking the patient to examine it closely. He produced it again at the end of the session, but the patient was still unable to recognize it. This procedure was repeated with the same result for nine sessions.

At the end of the next and final session, Zangwill again produced the pen and the patient again merely stared at it blankly. In desperation, Zangwill asked "Do you know who I am?", whereupon the patient replied, "Oh, yes. You're the man with all those fountain pens."

All these topics (except Zangwill's patient) are covered in massive detail in *Vision and Visual Dysfunction*. Most of the volumes are highly technical and assume a specialized knowledge of the area covered, whether it be "Spatial Vision", "Binocular Vision" or "Limits of Vision". Only a few of the volumes contain an introductory chapter setting the scene and outlining the main problems. There is, moreover, an enormous amount of repetition, partly because each volume is complete in itself; even within volumes, however, the same topics are repeatedly covered by different authors. For example, the distinction between the dysphonetic and the eidetic type of dyslexia is defined several times in the volume on "Vision and Visual Dyslexia". The general editor, J. R. Cronly-Dillon, seems to have left the format of the individual volumes very much up to their editors. Some contain glossaries, some do not: they are badly needed as there are a vast number of neologisms ranging from "heautoscopy" to "GOMS models". One of the most annoying tricks is the constant use of initials. Try digesting this sentence: "A subset of the tuned excitatory neurones TN and TS . . . give similar excitatory responses to GFS and RDS." To make matters worse, initials rarely appear either in the indices or glossaries. Unfortunately, none of the sections on reading describes experiments comparing reading speed for initials with that for terms set out in full — an exercise

Readings on cognition

■ **Brain Development and Cognition: A Reader** edited by Mark H. Johnson claims to be "the first comprehensive review of the emerging interface between cognitive neuroscience and the study of cognitive development". It contains both classic articles (some specially updated by their authors) and commissioned chapters. Blackwell, £60 (hbk), £19.99 (pbk).

■ **Frontiers in Cognitive Neuroscience** edited by Stephen M. Kosslyn and Richard A. Andersen contains 55 articles that provide a foundation for examining how brain function gives rise to mental activities such as perception, memory and language. The readings are grouped in parts that cover vision, auditory and somatosensory systems, attention, memory and higher cortical functions. The editors provide an introduction to each section. MIT Press, \$70.

that is badly needed, but the results of which might be rendered unintelligible because of the invention of a host of new initials by the experimenters.

Each volume is written by about 20 or 30 contributors, all of whom are experts in their specialized field. They achieve a high level of accuracy and are ruthless in their determination not to omit anything that might be relevant. As a source of reference for specialists, this book could hardly be bettered, though it is likely each will be able to cope only with the volume or volumes that fall within his or her speciality. There is, however, one volume that is an exception, because it can be read for pleasure as well as enlightenment. It differs from the rest in that it is written in its entirety by two authors, O. J. Grüsser and T. Landis. Although their first language is German, their writing is less turgid and more perspicuous than that of most of the other contributors. Apart from covering the obvious topics suggested by the title ("Visual Agnosias and Related Disturbances of Visual Perception and Cognition"), such as field defects, hemianopia, and prosopagnosia, they give readable introductions to psychological and neurophysiological theories and findings on almost every aspect of vision, including colour and motion perception, visual illusions, electroencephalography and eye movements. For light relief, they present a history of research on vision starting with Empedocles and proceeding through Galen, Avicenna, Kepler and Newton to Munk and Exner. Their penchant for history is also revealed in illuminating accounts of such matters as Bronze Age drawings and the development of script, starting with cuneiform. Unlike many of the other authors, who seem to be gritting their teeth while they grind through their allotted task, they write with a genuine enthusiasm, which has not precluded scholarship. Indeed, they are duly cautious in their interpretation of the many visual disorders they survey.

The volumes are sumptuously produced with a large number of diagrams and other illustrations, many in colour. The indices, on the other hand, are a disgrace. Few proper names are included — David Marr is in, Dave Hubel is out. Moreover, one searches in vain for many topics covered in the book. This lapse is particularly regrettable in a series that is primarily intended as a work of reference. Despite or perhaps because of the massive amount of information it contains, few workers will wish to own the whole set. It may be a feast, but it is a rather indigestible one. □

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Bonny baby

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Glaxo: A History to 1962. By R. P. T. Davenport-Hines and Judy Slinn. Cambridge University Press: 1992. Pp. 406. £55, \$110.

THERE can be few readers who would find this book in its entirety a riveting read. In their scholarly, deeply factual account of the early development of Glaxo, the world's second largest pharmaceutical company and the current role model for much of the industry, Davenport-Hines and Slinn provide not only the nuts and bolts of the story but the thread diameters to several decimal places. Unfortunately, because of the plethora of detail and despite some well-chosen illustrations, some sections make tedious reading: can one really appreciate the significance of "when in 1908 the Infant Welfare Centre at Rotherham began supplying Glaxo powder to mothers, 3,912 lbs was issued in the first sixteen months of the scheme" or the financial details of the fledgling Pakistan company in pounds and lakhs? On the other hand, detail can be fascinating, such as the personal characteristics of the principal players, in particular A. L. Bacharach, an early scientific recruit to Glaxo who proved a pervasive and positive influence throughout the organization. Or, for example, the consequences of company chairman Harry Jephcott's blunder in telling a visiting Merck executive that the anti-pernicious anaemia factor that both companies were attempting to isolate was pinkish red. As a scientist, I found that the tedious fractionally outweighed the fascinating; I could well imagine my business colleagues would say the reverse.

Be that as it may, this encyclopaedic effort has to be praised unreservedly for what it is: the definitive record of the origins of Glaxo from the antipodean

trading company founded by Joseph Nathan, a Jew from London's East End; through baby foods based on dried milk ('Glaxo', a euphonious sound-alike for 'Lacto' that proved acceptable to the Trades Mark Office), the first faltering steps into pharmaceuticals (vitamin products and antibiotics) and overseas markets (initially the British Commonwealth; belatedly, the crucial markets of Europe, North America and Japan), and the important acquisitions (Allen and Hanburys, Evans Medical); to the status of a leading British company, which Glaxo had become by 1962.

Those who, like me, would seek in such a book clues to the success of the present-day Glaxo will find Chapter 8, "Research and development: a strategy of science", the most revealing. The early lessons learnt during the development and marketing of vitamin B₁₂, various antibiotics, corticosteroids, vaccines and sundry minor medicines were used to shape strategies that were implemented by men of true professional quality, such as Jephcott, Henry Tizard (board member) and Tom Macrae (research director). The essence of their pragmatism can be

gleaned from statements such as "Committees can never take action successfully and effectively. In the finality individuals have to do whatever is to be done" (Jephcott), "The ideal director of research . . . is a strategist first and foremost. . . . He must inspire his own staff with the joy of practical achievement. He must deal gently but firmly with accountants who do not like doing what has never been done before. . . . It is not at all necessary that he should be a distinguished scientist, though he must be or must have been an experimenter" (Tizard) or "My God was that 10% stretched" (Macrae, referring to the board ruling that 10% of the research department's total income could be spent on whatever it liked).

It is not implausible that such attitudes were spliced firmly into the corporate Glaxo genome and that their expression over the years has been fundamental to Glaxo's success. However, the Glaxo Group of 1993 is very different in size, structure and organization from that of 1962; a second nuts-and-bolts story of a very different set of personalities and circumstances will be required for any such genetic traits to be discerned. □

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IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Growth business — Glaxo advertisement at King's Cross, London, in 1921.

Glaxo Holdings plc

Unborn interest

Mary Warnock

Life Before Birth: The Moral and Legal Status of Embryos and Fetuses. By Bonnie Steinbock. *Oxford University Press*: 1992. Pp. 256. £22.50, \$29.95.

It is a generalization widely accepted that what happens in the United States happens in the United Kingdom and elsewhere in Europe some years later. Much of Bonnie Steinbock's excellent book is taken up with discussions of US legal cases concerned with such issues as liability for prenatal injury, liability for wrongful prenatal death and even the suing of mothers by infants for wrongful life. These cases may seem of purely local interest, but it is not so. We are all increasingly prone to look to the courts to settle moral issues, including those that turn on the status of the unborn. Yet the law itself needs a firm and consistent moral base on which to found its judgements; there is little in the way of precedent to guide it, and much in the

way of prejudice and dogma to blind it.

Steinbock sets out to provide just such a base. Her first chapter is therefore philosophical, arguing for certain fundamental principles. In the subsequent chapters she shows the application of these principles in practice. In her introduction she kindly says that those who do not care for philosophy need not read Chapter 1. But it would be a great pity to omit it. It is clear, persuasive and almost entirely free of jargon.

The principles underlying Steinbock's moral attitudes, which she also urges her readers to adopt, are straightforward. Morality consists, she argues, of the taking into account by each of us of the interests of others than ourselves. Without such a reference to interests there would be no morality. It follows that all creatures capable of having an interest are a part of the moral world. They have a moral status, and cannot be used without consideration of their own good as well as the good of the person using them. Steinbock argues that to have an interest, a creature must be conscious. For being conscious entails having not merely needs that must be satisfied (as plants may have needs) but also wants, goals of which the creature is aware and which may be reached or frustrated. Because the creature experiences these wants, it has a stake in what happens. It makes sense to talk about doing something or refraining from it for the creature's sake.

There may be some dispute about whether animals other than humans can be said to have interests. In my view it is totally plausible to link interests to consciousness, provided that we do not take this to entail either that animals have rights (which must be created by law) or that their interests are to be weighed as equal to those of humans. Adopting these premises, then, we can argue that unborn embryos and fetuses do not have interests, because they are not conscious; and that their position is radically different from that of any child that has been born, except a child born with no brain or a brain so severely damaged that the child is not conscious.

Unborn embryos and fetuses, according to this argument, do not have the moral status that people who have been born are accorded. And so arguments against abortion, or the use of embryos for research, which turn on the interest or moral right of a fetus to life, or on an embryo's interest in not being so used, will all fail.

However, if this were all, there would be a plain argument for euthanasia for patients in a persistent vegetative state, for they are certainly not conscious. That this still seems to many people to be controversial is explained by the suggestion that people's interests, if they

have once had them, may seem to survive them, at least for a time. Just as we are reluctant to go against the wishes of the dead, so we continue to treat unconscious patients as if they were conscious.

Again, we feel inclined to argue that research using human embryos should not be just any research: it must be of genuine value, and not frivolous. Steinbock draws the distinction between having moral status (that is, being inevitably part of our moral considerations) and having moral value. All kinds of things, such as trees or landscapes or works of art, may have moral value and ought not to be destroyed wantonly. So unborn fetuses and embryos have moral value. They have a symbolic value — they stand for human life, and we may express our feeling of love or respect for humanity in their person. To use them frivolously or wantonly would, in Steinbock's view, be like burning the flag. This seems to me an important insight. That it is a matter of sentiment not reason makes it no less central to our morality.

Steinbock does not claim to have put forward original philosophical views in this book. But it is worthwhile, if only because it is consistent, thoroughgoing and intelligible. □

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Ethics and research

■ A second edition of William Paton's highly acclaimed *Man and Mouse: Animals in Medical Research* has just been issued in paperback. First published nine years ago, the book is now updated to take account of recent developments, including initiatives in toxicity testing. There is also an expanded discussion of the ethical problems surrounding animal experimentation and an appendix on the antivivisection movement. Oxford University Press, £7.99.

■ *Animal Experimentation and the Future of Medical Research*, edited by J. H. Botting, is a slender paperback containing essays on the advances in medical research that have depended on experimental work on animals. Contributors include Lord Adrian, D. K. Peters, D. Rees, S. Brenner, Sir John Vane, D. Hubel and Sir Walter Bodmer. The volume is based on a meeting organized by the Research Defence Society in 1991 at the Royal Society, London. Portland, £16.95.

■ *Embryo Experimentation: Ethical, Legal and Social Issues*, edited by Peter Singer *et al.*, is an accessible compendium of ideas and arguments on the subject first published in 1990 and now issued in paperback. In reviewing the volume in *Nature* (349, 375; 1991), A. Holland wrote: "The book as a whole mounts as effective an ethical case for embryo research as has yet been made, not least through its careful dismantling of the grounds for objection." Cambridge University Press, £11.95, \$17.95.

Worlds apart

Clifford A. Pickover

Mirror Worlds. By D. Gelernter. *Oxford University Press*: 1992. Pp. 237. \$24.95, £19.95 (hbk); \$12.95, £9.95 (pbk).

Glimpses of Heaven, Visions of Hell. By B. Sherman and P. Judkins. *Hodder and Stoughton*: 1992. Pp. 224. £12.99 (pbk).

Virtual Worlds. By B. Woolley. *Blackwell*: 1992. Pp. 274. £16.95, \$19.95.

THE concept of 'virtual reality' or 'cyberspace' is everywhere — from science-fiction films, museums and amusement arcade games, to serious computer journals. Certainly, computers of the future will allow us to enter lifelike environments. Already one can gaze into a computer-generated three-dimensional world whose verisimilitude is governed by the speed with which the computer can change its images in response to one's eye and head movements. The degree to which cyberspace becomes indistinguishable from reality depends partly on the development of new ways for humans to interact physically with computers by using, for example, speech-recognition devices.

Of these three fascinating books on virtual reality, *Mirror Worlds*, by David Gelernter, associate professor of computer science at Yale University, is the most unusual and perhaps most speculative. He doesn't so much focus on the traditional notions of virtual reality and its hardware as on the concept of a 'mirror world' — a huge true-to-life mirror image of corporations and ideas trapped inside a computer. For example, using a desktop computer and computer graphics, a hospital administrator might 'wander' through an entire medical complex. The hospital mirror world has a software version of every patient, doctor, bed and room and every important abstract entity: drug orders, cash in the bank and so on. Most of Gelernter's book describes how users will consult the mirror world like an encyclopaedia. He points out that many computer programs today run continuously and that this will become our basic way of thinking about programs: as continuously running factories, information refineries, operating around the clock gathering information from vast public repositories and chattering together. The basic idea is that mirror worlds can and will exist:

The real software revolution won't have much to do with fancy robots, computers in education, canned "multimedia" hype or the other hot topics that dominate this month's hit parade. It will center instead on software that steps over the crucial boundary between private and public.

In *Glimpses of Heaven, Visions of Hell*, Barrie Sherman and Phil Judkins take the reader on a comprehensive and equally absorbing tour of the political, economic, social, moral, psychological and religious aspects of virtual reality. In the authors' view, the implications will be far-reaching: sex will never be the same and virtual reality will almost certainly be exploited by criminals.

By contrast, Benjamin Woolley, author of *Virtual Worlds*, focuses on the dramatic intellectual and cultural upheavals that gave birth to virtual reality, and the people who have promoted it. Some may find his style irritating, however:

The gloomy postmodernists think that rationalist values of truth and certainty are being destroyed. The upbeat, bullshitting virtual realists think they can make something better, that dreams can truly be turned into reality But is reality really dead? Are we obliged to leave it to the virtual realist to create another one for us? Must we sink into postmodern autism?

But there is much else of substance in the book for those who shy away from this kind of wild philosophizing. □

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Wire taps

Orlando Belpaese

The Bohr–Einstein Transcripts. Edited by T. J. Gschärtlhuber. Midgley–Cideryard: 1993. Pp. 137. \$14.95 (pbk).

THE demise of the art of letter writing with the advent of the telephone is often regretted, especially by historians robbed of valuable primary material. Had Darwin had an early telephone, we would have been deprived of his ruminations on the development of the theory of evolution, which can be gleaned



Einstein — ringing true?

from his published correspondence.

The most celebrated scientific debate of this century was that between Einstein and Bohr, conducted mostly by letter and at conferences, but also in the *Physical Review* and other learned journals. Not only have Einstein's difficulties with quantum mechanics, despite his seminal part in its invention, cast a long shadow over the development of physics in the latter part of this century; they have also kept an army of historians employed, poring over the protagonists' extensive correspondence.

Curiously, the Bell Telephone Company helped preserve another small corner of the quantum debate, now presented in this handsome but diminutive booklet prepared by Jerry Gschärtlhuber. At about the time Einstein arrived at Princeton, in 1933, Bell was working on a prototype telephonic recording machine, the Mnemophone. A wire recorder that could be attached to a telephone, this was in many ways a precursor to the modern answerphone (it is often forgotten that many devices, and prototypical facsimile transmitters, were tested before the turn of the century).

As part of a promotional effort, Bell made gifts of a few Mnemophones to celebrities of the time — President Roosevelt, Sinclair Lewis, George Gershwin, Charles Lindbergh and, as the ultimate symbol of the new science, Einstein. Einstein was naturally en-

chanted by this machine — not merely an ex-patent clerk, he had also dabbled in inventions himself, and had patents on, among other things, a hearing aid and a noiseless refrigerator. He recorded few of his telephone conversations with the Bell machine, which was evidently unreliable (according to Gschärtlhuber, Einstein can be heard cursing and even striking the malfunctioning device at times), but he clearly treasured his occasional transatlantic discussions with Bohr enough for these to form the major part of the surviving magnetic archive.

Gschärtlhuber has faithfully transcribed and translated these conversations (the originals, of course, were in German), released to him by the Einstein Archive at Princeton University, in full, including even the everyday trivia of all phone calls — talk of the weather, the Princeton Tigers' recent games, vacation preparations and so on. Inevitably, the quantum debate has none of the coherence evident in the Bohr–Einstein correspondence (recorded by Bohr in *Albert Einstein: Philosopher–Scientist*, Tudor, New York, 1949), but key ideas are instantly recognizable.

More significantly, historians of science have already been excited by hints of a lost paper (*Über die Elektrodynamik und die fermische Theorie der hochenergetischen Betastrahlen*) tying in Fermi's new theory of nuclear β -decay with electrodynamics. Possibly a precursor to the electroweak theory of the 1960s and 70s, the paper might give the lie to the idea that Einstein's late work was blocked by an obsession with unifying electromagnetism only with gravity. From the conversations, Bohr evidently read, disliked and possibly killed the ideas. The hope is that, although no version of this paper survives among Einstein's possessions, a draft may still be in Bohr's archives, waiting to be found.

Immaculately prepared and annotated, and with rare photographs of both scientists, this book will not change our view of the developing philosophy of quantum mechanics; but it casts the debate into a charmingly humane light. Many will be amused by the appendix, containing recorded conversations with several Hollywood starlets, major and minor, of the day, which make a delightful addition to Einstein's famous collection of autographs. And historians who fear that the arrival of mobile phones will rob us even of the fruits of answerphone messages can take heart from recent revelations of misdeeds involving British royalty. □

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The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation

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The mossy fibre pathway in the hippocampus uses glutamate as a neurotransmitter, but also contains the opioid peptide dynorphin. Synaptic release of dynorphin causes a presynaptic inhibition of neighbouring mossy fibres and inhibits the induction and expression of mossy fibre long-term potentiation. These findings demonstrate a physiological role for a neuropeptide in the central nervous system, provide a functional basis for the coexistence of a neuropeptide with classic neurotransmitters and demonstrate the very different roles played by these two classes of signalling molecules.

THE discovery that a variety of small peptides are specifically present within neurons that also contain classic neurotransmitters has led to the concept of coexistence of transmitters¹. The functional implications of this concept have been most thoroughly developed in the vertebrate peripheral nervous system²⁻⁴. It has been shown in the peripheral nervous system that the action of peptides differs from that of classic transmitters in that high-frequency stimulation is required for peptide release, their action is long lasting and they act at considerable distances from their site of release. The widespread occurrence of the colocalization of peptides with classic neurotransmitters within neurons of the central nervous system (CNS) has led to the assumption that the model developed in the peripheral nervous system also applies to the CNS. The functional correlates of coexistence in the CNS, however, have been difficult to establish unambiguously, as indeed has any role for peptides as physiological messengers in the CNS.

We have used a well characterized monosynaptic pathway, the mossy fibre pathway in the hippocampus, to test for any physiological role of peptides. The fibres in this pathway contain and release the amino acid glutamate^{5,6} and opioid peptides, in particular dynorphin⁷⁻¹¹. We find that activation of mossy fibres, in addition to generating the well studied glutamatergic fast excitatory postsynaptic potential (e.p.s.p.), also generates a long-lasting inhibition of neighbouring mossy fibre synapses by a presynaptic action of synaptically released dynorphin. Brief tetani, which are below threshold for mossy fibre long-term potentiation (LTP), generate LTP in the presence of opioid receptor antagonists. Furthermore, the presynaptic inhibition by dynorphin is more pronounced on synapses expressing LTP. These results establish dynorphin as a synaptic transmitter at glutamatergic mossy fibre synapses and demonstrate an important interaction between this peptidergic system and the mechanisms governing the generation and expression of LTP.

Dynorphin inhibits synaptic transmission

We have used extracellular recording techniques throughout this study to record the mossy fibre input to CA3 pyramidal cells (see legend to Fig. 1). The typical mossy fibre field potential recorded in stratum lucidum (Fig. 1A) is shown in Fig. 1B which shows marked facilitation when the fibres are stimulated a second time (paired-pulse facilitation). Bath application of dynorphin (100–500 nM) strongly inhibited mossy fibre synaptic responses (Fig. 1Bb). In contrast to previous studies, which reported a variable effect¹² or no effect¹³, we found the effect of dynorphin to be highly reproducible, with inhibition occurring in 49 out of 51 slices examined and averaging $40 \pm 2\%$ ($n = 51$) (Figs 2a, 3b and 6). Figure 1Bc shows that the non-NMDA (*N*-methyl-D-aspartate) glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) blocks much of the field potential, consistent with the conclusion that mossy fibres release glutamate^{5,6}. A considerable presynaptic fibre volley response remains, however, and the relative contribution of this presynaptic fibre volley varied considerably in different slices. Therefore, at the end of each experiment CNQX was applied and the remaining fibre volley was then subtracted from all the records (Fig. 1Bd).

The specificity of the action of dynorphin was tested by comparing in the same slice the responses to stimulation of associational-commissural (assoc-com) afferents in stratum radiatum and mossy fibres. The depressant effect of dynorphin was entirely selective for mossy fibre responses (Fig. 2A). This figure also shows that the response to dynorphin was slow in onset and long-lasting, typically requiring 30–60 min for recovery. Brief puff application of 5 μ M dynorphin in s. lucidum, but not at the site of stimulation in the granule cell layer, resulted in an inhibition which lasted 10–15 min ($n = 3$). The long duration of action seemed to be due to the slow washout of the peptide, because rapid application of opioid antagonists completely reversed the depression within 5 min ($n = 6$). We then examined the action of dynorphin on intracellularly recorded mossy fibre e.p.s.ps (Fig. 2b). Dynorphin decreased the peak amplitude and the rate of rise of the e.p.s.ps ($25 \pm 8\%$ for rate of rise, $n = 6$). In addition, dynorphin decreased the inhibitory postsynaptic potential (i.p.s.p.) that followed the e.p.s.ps when the i.p.s.ps were evoked by mossy fibre activation (Fig. 2b). The effects of dynorphin were reversed in each case by the opioid antagonist naloxone (Fig. 2b), indicating that the action was mediated by opioid receptors. Dynorphin had no consistent effect on membrane potential, input resistance or the threshold for action potential generation in CA3 pyramidal cells.

Dynorphin activates kappa 1 opioid receptors

Experiments were done to characterize the receptor subtype responsible for the dynorphin inhibition of mossy fibre responses. On the basis of the action of selective agonists and

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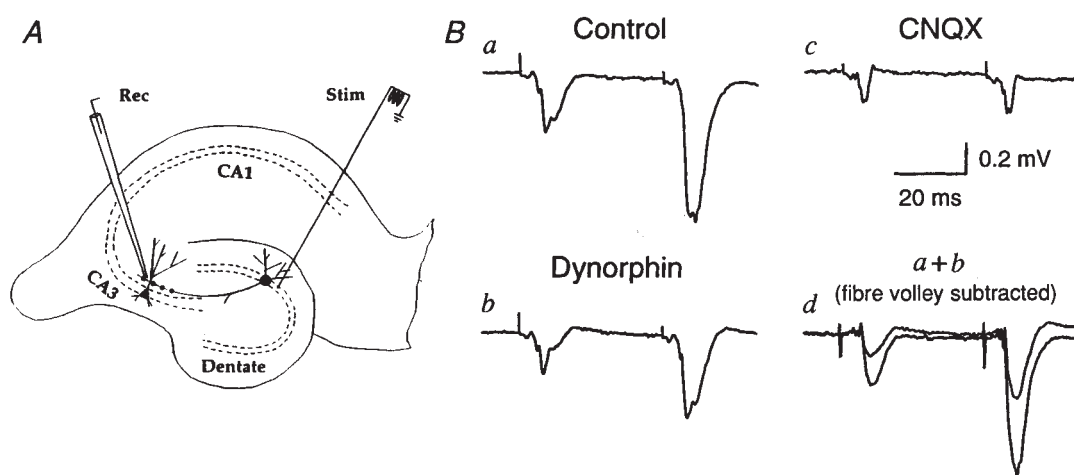
antagonists, opioid receptors have been divided into three types: mu, kappa and delta¹⁴. Consistent with the idea that dynorphin is a selective kappa agonist¹⁵, we found that the selective kappa antagonist nor-binaltorphimine (nor-BNI)¹⁶ (100 nM) completely blocked the inhibitory effect of dynorphin ($n=4$). Nor-BNI at these same concentrations had no effect on the well characterized mu¹⁷⁻¹⁹ and delta²⁰ receptor-mediated disinhibitory action in the CA1 region caused by the selective agonists DAMGO²¹ (100 nM) ($n=3$) and DPDPE²² (1 μ M) ($n=3$), respectively. And it did not have any effect on membrane potential or input resistance ($n=4$). Furthermore, concentrations of dynorphin that depressed mossy fibres had no disinhibitory action in the CA1 region ($n=3$), indicating that dynorphin is not crossing over to activate mu or delta opioid receptors. These results strongly suggest that the inhibition of mossy fibre responses is mediated by kappa receptors. We have also found that U69,593 (300 nM) ($n=5$), a kappa agonist that has been used to define kappa 1 sites^{23,24}, depressed mossy fibre responses, an effect that was blocked by nor-BNI. The depression ($43 \pm 4\%$) was equivalent to that seen with dynorphin and subsequent application of dynorphin produced no further depression. These findings with U69,593 and with nor-BNI which is a kappa 1 selective ligand²⁴ are somewhat surprising in that, although kappa binding sites are abundant in *s. lucidum* of the guinea-pig⁹, kappa 1 binding sites have not been detected¹⁰. The rat, in contrast to other rodent species, shows little kappa receptor binding in *s. lucidum*⁹. These physiological results indicate

that kappa 1 receptors are present in the mossy fibre pathway of the guinea-pig which, when activated, depress synaptic transmission.

Dynorphin could depress synaptic transmission by blocking the postsynaptic action of glutamate or by blocking the release of glutamate. To distinguish between these possibilities we compared the effects of dynorphin on responses to glutamate applied in *s. lucidum* from a double-barrelled pipette and to mossy fibre synaptic responses, both recorded from the adjacent barrel. Although dynorphin had its typical depressant action on the mossy fibre synaptic response it had no effect on the glutamate response (Fig. 3a). Both the synaptic and glutamate responses, however, were susceptible to CNQX applied to the bath (Fig. 3a). This differential action of dynorphin is summarized for four slices in Fig. 3b. The lack of effect of dynorphin on the responses to exogenously applied glutamate is consistent with a presynaptic action.

Paired-pulse facilitation is a phenomenon seen at many chemical synapses, including mossy fibre synapses (Fig. 1B), and reflects a presynaptic enhancement in transmitter release²⁵. Manipulations that decrease transmitter release increase paired-pulse facilitation²⁶⁻²⁸. We therefore examined the effects of dynorphin on paired-pulse facilitation. In these experiments the stimulus strength was increased so that the amplitude of the response to the first pulse in the presence of dynorphin matched the amplitude of the control response. An example is illustrated in Fig. 3c which shows that during the action of dynorphin the

FIG. 1 Diagram of hippocampus and effect of dynorphin on mossy fibre field responses. A, Diagram showing the placement of the stimulating electrode in the dentate granule cell layer and the extracellular recording electrode in *s. lucidum*. B, Sample records of mossy fibre field potentials. Paired-pulse stimulation (40 ms interval) showed large facilitation, as is typical of mossy fibre inputs. Ba is before and Bb is 10 min after the addition of dynorphin (500 nM) to the Ringer's solution. Bc shows the response 10 min after the addition of CNQX (20 μ M) and Bd shows the superimposition of pre- and postdynorphin traces after subtracting the fibre volley contamination of each. Each record is the average of 5 to 10 traces.



electrode was then placed at this site, some minor adjustments during intracellular recordings were occasionally still necessary to improve further the mossy fibre e.p.s.p. For field recordings, the restricted anatomy of the mossy fibre input to *s. lucidum* as well as the reversal of the waveform as the recording electrode is moved from *s. lucidum* to *s. radiatum* serve to define mossy fibre inputs clearly. At the end of all field experiments, 20 μ M CNQX or occasionally DNQX was added to the bath to assess the fibre volley component of the response. This component could often be a large fraction of the e.p.s.p. in response to the first of paired stimuli, especially after manipulation-induced depression. Therefore, we analysed the peak amplitude of the second response throughout this study unless otherwise noted (see Fig. 6) because the larger synaptic component allowed for more accurate and less variable measurements. When an associational-commissural input was used, a second stimulating electrode in CA3 was placed in *s. radiatum* to the CA1 side of the recording electrode and far away from *s. lucidum* to avoid activating mossy fibres. Baseline transmission was monitored at 0.05 Hz with paired pulses given 40 ms apart. A normal LTP-inducing stimulus consisted of 4 trains of 100 Hz stimulation for 1 s, separated by 20 s and given at baseline stimulus strength. All tetani were given in the presence of 20 μ M D-APV to block NMDA receptor-dependent, non-mossy fibre LTP. All values are expressed as means $\pm$ s.e.m. Drugs used were dynorphin A (1-17) (Bachem); nor-Binaltorphimine 2HCl, CNQX, DNQX (Research Biochemicals); D-APV (Cambridge Research Biochemicals); naloxone HCl (Endo Labs); DAMGO, DPDPE, U69,593 (Sigma).

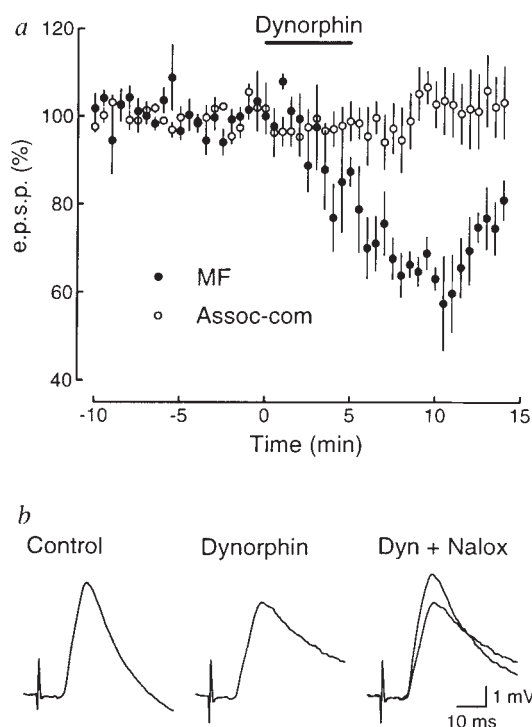


FIG. 2 Inhibitory action of dynorphin on mossy fibre responses. *a*, Concurrent recordings of mossy fibre (MF; closed symbols) and associational-commisural (assoc-com; open symbols) field potentials from the same slice ($n=5$). The MF inputs were strongly inhibited by a brief application of dynorphin (100–250 nM; solid bar), whereas the initial slope of the assoc-com inputs were unaffected. *b*, Intracellular recordings of MF responses were also depressed by dynorphin (500 nM) and this was reversed by the opioid receptor antagonist naloxone (10 μ M) (Nalox), applied for 7 min. The postdynorphin e.p.s.p. is shown superimposed on the postnaloxone e.p.s.p. for reference (Dyn + Nalox). Each record is the average of 20 to 30 traces.

facilitation evoked by paired pulses is enhanced. The results from 10 slices showed that the inhibition is associated with a $17.7 \pm 7.5\%$ increase in facilitation ($P < 0.05$, two-tailed paired sample Student's t test). This finding strongly suggests that kappa receptors are present on mossy fibre synaptic terminals and that their activation reduces glutamate release. The selective disinhibitory action of dynorphin on mossy fibre responses (Fig. 2*b*) could also be explained by an action on presynaptic kappa receptors on the terminals of mossy fibre collaterals. Our findings agree with many other studies indicating a presynaptic location of kappa receptors^{29–34}.

Dynorphin mediates heterosynaptic depression

We next considered whether the opioid peptides present in the mossy fibres have a physiological role in this pathway. Application of naloxone had no effect on e.p.s.ps evoked by low-frequency stimulation (0.05 Hz) in five of seven slices and had no effect on the frequency potentiation that occurred when the

stimulus rate was increased from 0.05 to 1 Hz stimulation (potentiation in control = $285 \pm 29\%$, $n=7$; potentiation in naloxone = $292 \pm 38\%$, $n=7$). These results suggest that significant amounts of peptide are not released from these synapses at frequencies of up to 1 Hz. We wondered, therefore, whether higher stimulus frequencies might be necessary for peptide release. Indeed, when one of two independent mossy fibre pathways is tetanized, a transient depression lasting 10–20 min is seen in the untetanized pathway^{35,36}. An example of this heterosynaptic depression is shown in Fig. 4*a*. One possible mechanism for this depression is that dynorphin is released from the tetanized synapses and acts on nearby synapses. Therefore, the naloxone sensitivity of this depression was examined. Two groups of slices, with ($n=8$) or without ($n=15$) naloxone, were compared. Naloxone blocked the heterosynaptic depression and actually unmasked a small facilitation that probably reflects a small subset of fibres in the control pathway that were also activated by the tetanus to the other pathway (Fig. 4*b*).

FIG. 3 Dynorphin decreases transmitter release from mossy fibre synapses. *a*, Sample records of responses to mossy fibre stimulation (MF) or iontophoretically applied glutamate (Glu; solid bar) evoked alternately in the same slice before (Control) and 10 min after (Dynorphin) the addition of dynorphin (500 nM) to the Ringer's solution. A double-barrelled micropipette was used, one barrel for recording and the other for iontophoretic application of glutamate, to ensure that glutamate was applied to the recording site. The recording barrel contained 3 M NaCl and the other barrel was filled with glutamate (200 mM in distilled water, pH 8) for iontophoresis (400 nA, 100 ms, no retaining current). MF and glutamate (Glu) sample records are averages of 15 and 5 responses, respectively. Recordings obtained after addition of 20 μ M CNQX to the Ringer's solution are superimposed on the postdynorphin traces (CNQX). *b*, Comparison of responses in the same slice to mossy fibre stimulation (evoked; filled symbols) and iontophoretically applied glutamate (100–800 ms, 200–800 nA, no retaining current; open symbols) in *s. lucidum*. Dynorphin (500 nM; solid bar) depressed the evoked responses, but had no effect on the iontophoretic responses ($n=4$). *c*, Increase in paired-pulse facilitation after dynorphin. Sample records are averages of 5 traces before (control) or 10 min after the addition of dynorphin (500 nM) to the Ringer's solution. The fibre volley was subtracted from both responses.

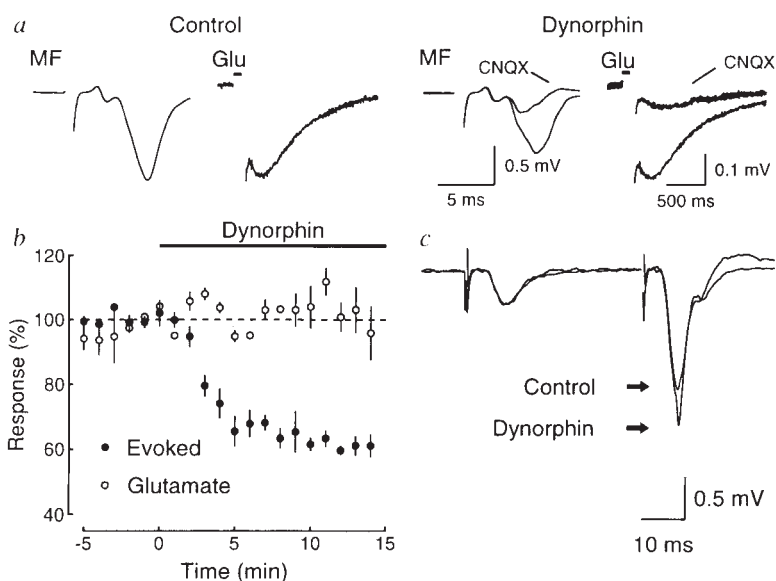
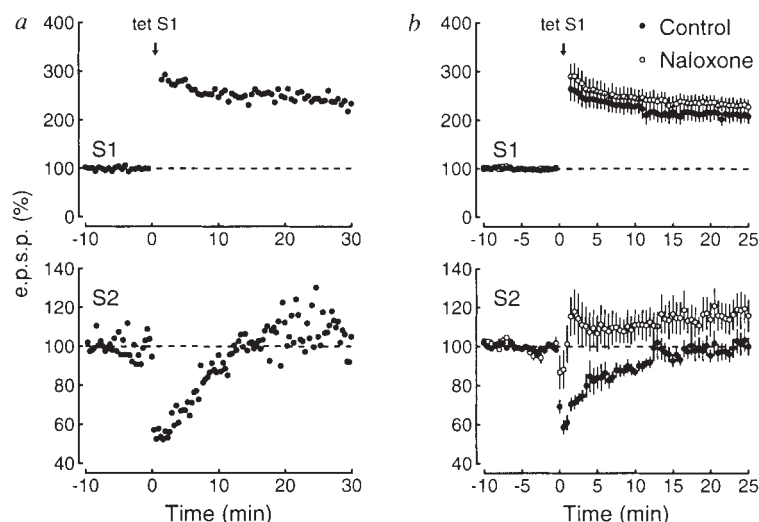


FIG. 4 Heterosynaptic depression of mossy fibre responses is blocked by the opioid receptor antagonist naloxone. *a*, A tetanus (100 Hz for 1 s repeated 4 times at 20 s intervals) was given at test stimulus strength to one pathway (S1) at time 0. The responses to this input exhibit LTP. Monitored concurrently were the responses to a second input that did not receive any stimulation during the tetanus to the other pathway (S2). These responses exhibit a transient depression. *b*, Graph of tetanized (S1) and tetanized (S2) inputs in a group of control slices (filled symbols; $n=15$) and a group of slices that had naloxone (10 μM) added to the bath (open symbols; $n=8$). LTP is seen in both cases, but the transient depression is blocked by naloxone. The typical decaying component to mossy fibre LTP^{36,38} is not apparent in these figures because the second of paired pulses is plotted. In all experiments, two pathways that did not show any across-pathway paired-pulse facilitation were chosen to minimize the number of fibres common to both inputs.



The long duration of the inhibition is probably due to the slow clearance of peptide because rapid application of antagonists reverses the action of dynorphin within 5 min.

Dynorphin inhibits mossy fibre LTP

Because mossy fibre synaptic transmission is strongly modulated by release of endogenous dynorphin, we wondered if dynorphin normally plays a role in mossy fibre LTP. The mossy fibre LTP induced by the tetanus used to examine heterosynaptic depression (in the presence of the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV)) was not blocked by naloxone, contrary to a previous report³⁷, and may actually have been enhanced to a small extent (Fig. 4*b*), although not as much as might be expected if dynorphin were also causing a homosynaptic depression. Perhaps during the early intense potentiation the inhibitory action is masked. We have specifically tested the possibility that synaptically released dynorphin may act homosynaptically and inhibit the induction of mossy fibre LTP, by selecting a tetanus protocol (5–10 pulses at 100 Hz repeated at 20-s intervals 4 times) that in most cases failed to generate LTP. Nor-BNI was then added to the bath, which had no effect on baseline transmission ($+2.6 \pm 2.7\%$, $n=11$, $P>0.05$), and the same stimulus was repeated. In the presence of nor-BNI, LTP could be evoked by the same tetanus that initially failed to evoke LTP. A summary graph of 11 experiments is shown in Fig. 5*a*. In a separate set of control slices a second tetanus was given in

the absence of nor-BNI and no LTP was seen (Fig. 5*b*). This ensures that the LTP shown in Fig. 5*a* resulted from the action of nor-BNI. In 10 of the experiments illustrated in Fig. 5*a*, a second pathway was monitored and the heterosynaptic inhibition observed in this pathway was blocked by nor-BNI. The results presented above indicate that synaptically released dynorphin can prevent the induction of mossy fibre LTP.

Previous studies have proposed that the expression of mossy fibre LTP, which is independent of NMDA receptors, may be mediated by an increase in glutamate release because paired-pulse facilitation is decreased after LTP induction^{38,39}. We therefore wondered whether synapses that had undergone LTP might have an altered sensitivity to the action of dynorphin. In this experiment two independent pathways were examined. After LTP had been evoked in one pathway the stimulus strength was decreased so that the response was similar in size to the control pathway. Under these conditions the tetanized pathways were on average potentiated to $294 \pm 39\%$ (measured at 10 to 15 min) of control. Dynorphin was then applied and the inhibition compared in the two pathways. As shown in Fig. 6, which summarizes the results from eight experiments, dynorphin produces a larger inhibition of synapses expressing LTP ($18.3 \pm 4.9\%$, $P<0.05$, two-tailed paired sample Student's *t* test). This result is of interest for two reasons. First, the action of dynorphin depends on the prior activity of the synapse. Second, studies on the mechanism of action of dynorphin in a variety of systems

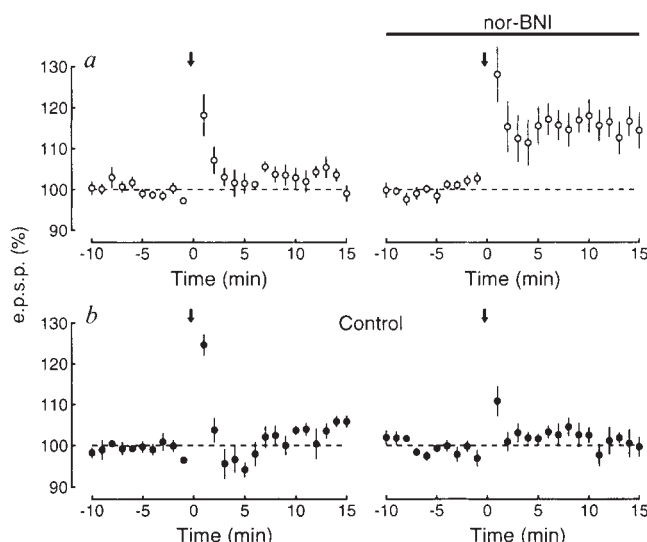
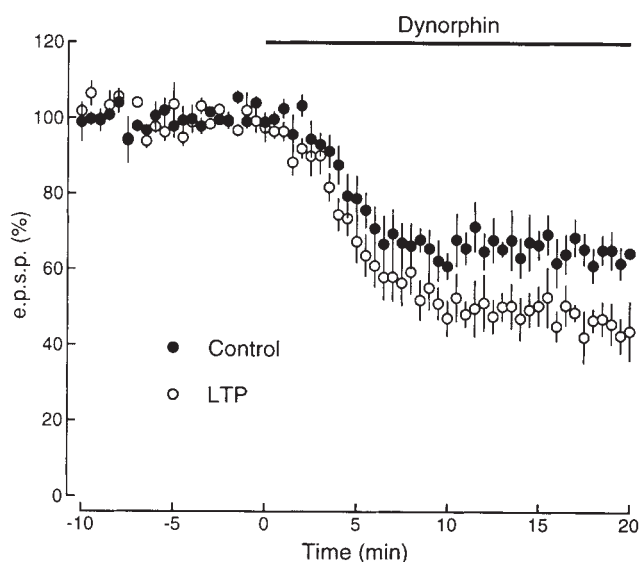


FIG. 5 Synaptically released dynorphin blocks the induction of mossy fibre LTP. *a*, After baseline responses were stable for 10 min, a series of brief tetani (5–10 impulses, 100 Hz, repeated 4 times at 20 s intervals) were presented at the test stimulation strength. If test responses, averaged between 5–15 min after this stimulation, deviated $<10\%$ from prestimulation levels, then the stimulation was accepted as subthreshold for generation of LTP. If larger than 10% the experiment was excluded. Nor-BNI (100 nM) was then applied to the bath. In the presence of nor-BNI the identical stimulation now evokes LTP. *b*, In a separate group of 5 slices recorded in a manner identical to those in *a*, no nor-BNI was applied and the identical stimulation was given again after a 10 min stable baseline. The interval between the tetanus and the first subsequent test stimulus varied up to 30 s, thus the data points immediately following a tetanus are not an accurate representation of post-tetanic potentiation. Furthermore, points that exceeded the scale of the figure are not shown.

FIG. 6 Dynorphin is more effective on mossy fibre synapses expressing LTP. Two separate mossy fibre pathways in one slice were concurrently monitored. One of these was given a standard LTP-inducing tetanus and the other was not. Once responses stabilized (10–15 min), the stimulus strengths were adjusted so that the pathways gave roughly identical responses. After a stable 10 min baseline period, dynorphin (500 nM; solid bar) was applied and the responses of both the previously tetanized (open symbols) and untetanized (filled symbols) pathways were monitored ($n=8$). In this series of experiments, analysis of the second e.p.s.p. of a pair, although suggestive, did not show a convincing difference in sensitivity to dynorphin of tetanized and untetanized pathways. Thus it was necessary to analyse the first e.p.s.p. of a pair in order to demonstrate clearly an increased sensitivity to the effects of dynorphin. This more pronounced differential effect of dynorphin on the response to the first e.p.s.p. supports the notion that dynorphin is counteracting the effects of LTP (that is the enhancement seen with LTP is greater on the first pulse and the depression seen with dynorphin is greater on the first pulse).



have found it to have a highly specific presynaptic inhibitory action^{29–34}, apparently acting primarily on the N-type Ca^{2+} channel^{40–43}. Thus, the finding that dynorphin interacts with LTP is consistent with a model in which the expression of mossy fibre LTP may occur at the level of a presynaptic N-type Ca^{2+} channel which may play a role in synaptic transmitter release from mossy fibres⁴⁴.

Long-term potentiation has been proposed as a cellular mechanism for learning and memory. The inhibitory effects of dynorphin on mossy fibre LTP are quite consistent with behavioural studies indicating that opioid receptor activation impairs cognitive function and conversely naloxone enhances performance in a variety of learning and memory tasks^{45,46}. Specifically, it has been reported that the disruption of cognitive performance induced by electrical stimulation in the dentate gyrus is prevented by systemic administration of naloxone⁴⁷.

Discussion

This study has taken advantage of the anatomical simplicity of the hippocampal mossy fibre system and provides strong evidence that the opioid peptide dynorphin is a neurotransmitter

in this system. The requirement for high-frequency stimulation to release dynorphin, its long duration of action and its action at a distance are similar to the peptidergic transmission characterized in the peripheral nervous system^{2–4}. The relationship of this peptidergic system to LTP in these same synapses is intriguing. The relative requirements for peptide release and LTP are such that synaptically released dynorphin during a tetanus can inhibit the induction of LTP. Thus, dynorphin seems to regulate the threshold for LTP induction in this system, as well as produce a frequency-dependent lateral inhibition that could serve to focus potentiation into the CA3 region. These findings complement some theories of learning and memory that hypothesize that the CA3 region of the hippocampus is a site of associative memory formation⁴⁸. A focusing of inputs, such as that mediated here by dynorphin, is a critical requirement in such theories if the memory capacity is to be large and have minimal error. Finally, the greater sensitivity of synapses expressing LTP to the inhibitory action of dynorphin adds a conditional property to the action of dynorphin and also suggests that dynorphin and LTP may share a common step, perhaps an N-type Ca^{2+} channel. □

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- Hökfelt, T. *Neuron* **7**, 867–879 (1991).
- Jan, L. Y. & Jan, Y. N. *J. Physiol.* **327**, 219–246 (1982).
- Jan, Y. N. & Jan, L. Y. *Trends Neurosci.* **6**, 320–325 (1983).
- Lundberg, J. M. & Hökfelt, T. *Trends Neurosci.* **6**, 325–333 (1983).
- Storm-Mathisen, J. et al. *Nature* **301**, 517–520 (1983).
- Crawford, I. L. & Connor, J. D. *Nature* **244**, 442–443 (1973).
- Gall, C., Brecha, N., Karten, H. & Chang, K.-J. *J. comp. Neurol.* **198**, 335–350 (1981).
- McGinty, J. F., Henriksen, S. J., Goldstein, A., Terenius, L. & Bloom, F. E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 589–593 (1983).
- McLean, S., Rothman, R. B., Jacobson, A. E., Rice, K. C. & Herkenham, M. *J. comp. Neurol.* **255**, 497–510 (1987).
- Wagner, J. J., Evans, C. J. & Chavkin, C. *J. Neurochem.* **57**, 333–343 (1991).
- Ternan, D. M. et al. *Brain Res. Bull.* **21**, 343–351 (1988).
- Iwama, T., Ishihara, K., Satoh, M. & Takagi, H. *Neurosci. Lett.* **63**, 190–194 (1986).
- Caulfield, R. M. & Chavkin, C. *J. pharmac. Exp. Ther.* **252**, 1361–1369 (1990).
- Goldstein, A. *Trends pharmacol. Sci.* **8**, 456–459 (1987).
- Chavkin, C., James, I. F. & Goldstein, A. *Science* **215**, 413–415 (1982).
- Takemori, A. E., Ho, B. Y., Naeseth, J. S. & Portoghesi, P. S. *J. Pharmac. exp. Ther.* **246**, 255–258 (1988).
- Nicoll, R. A., Siggins, G. R., Ling, N., Bloom, F. E. & Guillemin, R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2584–2588 (1977).
- Ziegler, S., Wrenn, F., French, E. D., Siggins, G. R. & Bloom, F. E. *Science* **205**, 415–417 (1979).
- Neumaier, J. F., Mailheau, S. & Chavkin, C. *J. Pharmac. exp. Ther.* **244**, 564–570 (1988).
- Lupica, C. R. & Dunwiddie, T. V. *Synapse* **8**, 237–248 (1991).
- Gillan, M. G. C. & Kosterlitz, H. W. *Br. J. Pharmac.* **77**, 461–469 (1982).
- Mosberg, H. I. et al. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5871–5874 (1983).
- Nock, B., Giordano, A. L., Cicero, T. J. & O'Connor, L. H. *J. Pharmac. exp. Ther.* **254**, 412–419 (1990).
- Zukin, R. S., Eghbali, M., Olive, D., Unterwald, E. M. & Tempel, A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4061–4065 (1988).
- Zucker, R. S. *A. Rev. Neurosci.* **12**, 13–31 (1989).
- Katz, B. & Miledi, R. *J. Physiol.* **195**, 481–492 (1968).

- Mallart, A. & Martin, A. R. *J. Physiol.* **196**, 593–604 (1968).
- Creager, R., Dunwiddie, T. & Lynch, G. *J. Physiol.* **299**, 409–424 (1980).
- McFadzean, I., Lacey, M. G., Hill, R. G. & Henderson, G. *Neuroscience* **20**, 231–239 (1987).
- Gannon, R. L. & Terrian, D. M. *Brain Res.* **548**, 242–247 (1991).
- Kato, M., Chapman, C. & Bicknell, R. J. *Brain Res.* **574**, 138–146 (1992).
- Gauchy, C., Desban, M., Krebs, M. O., Glowinski, J. & Keme, M. L. *Neuroscience* **41**, 449–458 (1991).
- Pinnock, R. D. *Brain Res.* **583**, 237–246 (1992).
- Wagner, J. J., Caulfield, R. M. & Chavkin, C. *J. Neurosci.* **12**, 132–141 (1992).
- Bradley, J. E. & Barrionuevo, G. *Neuroscience* **35**, 265–271 (1990).
- Zalutsky, R. A. & Nicoll, R. A. *Neurosci. Lett.* **138**, 193–197 (1992).
- Martin, M. R. *Neuropeptides* **4**, 45–50 (1983).
- Zalutsky, R. A. & Nicoll, R. A. *Science* **248**, 1619–1624 (1990).
- Staubli, U., Larson, J. & Lynch, G. *Synapse* **5**, 333–335 (1990).
- Gross, R. A. & Macdonald, R. L. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5469–5473 (1987).
- Bean, B. *Nature* **340**, 153–156 (1989).
- Adamson, P., Xiang, J.-Z., Mantzourides, T., Brammer, M. J. & Campbell, I. C. *Eur. J. Pharmac.* **174**, 63–70 (1989).
- Xiang, J.-Z., Adamson, P., Brammer, M. J. & Campbell, I. C. *Neuropharmacology* **29**, 439–444 (1990).
- Kamiya, H., Sawada, S. & Yamamoto, C. *Neurosci. Lett.* **91**, 84–88 (1988).
- Gallagher, M. in *Opioids in the Hippocampus* (eds McGinty, J. F. & Friedman, D. P.) 118–132 (NIDA Res. Monograph, 1988).
- Decker, M. W. & McLaughlin, J. L. *Synapse* **7**, 151–168 (1991).
- Collier, T. J. & Routenberg, A. *Brain Res.* **310**, 384–387 (1984).
- Rolls, E. T. *Cold Spring Harb. Symp. quant. Biol.* **55**, 995–1006 (1990).
- Zalutsky, R. A. & Nicoll, R. A. in *Transmitter Amino Acid Receptors: Structures, Transduction and Models for Drug Development* Vol. 6 (Barnard, E. A. & Costa, E.) 415–422 (Thieme Medical, New York, 1991).

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Discovery of jovian dust streams and interstellar grains by the Ulysses spacecraft

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ON 8 February 1992, the Ulysses spacecraft flew by Jupiter at a distance of 5.4 AU from the Sun. During the encounter, the spacecraft was deflected into a new orbit, inclined at about 80° to the ecliptic plane, which will ultimately lead Ulysses over the polar regions of the Sun¹. Within 1 AU from Jupiter, the onboard dust detector² recorded periodic bursts of submicrometre dust particles, with durations ranging from several hours to two days, and occurring at approximately monthly intervals (28 ± 3 days). These particles arrived at Ulysses in collimated streams radiating from close to the line-of-sight direction to Jupiter, suggesting a jovian origin for the periodic bursts. Ulysses also detected a flux of micrometre-sized dust particles moving in high-velocity (≥ 26 km s⁻¹) retrograde orbits (opposite to the motion of the planets); we identify these grains as being of interstellar origin.

FIG. 1 Ulysses trajectory and geometry of dust detection—oblique view from above the ecliptic plane also showing the Sun and the orbits of Earth and Jupiter (in the foreground). Arrows indicate the flow of interstellar dust. The trajectory of Ulysses¹ after Jupiter closest approach (CA) is deflected into an orbit inclined at 80° to the ecliptic going south. Numbers along the trajectory refer to positions of Ulysses at which dust streams were detected—dotted lines point to Jupiter. Two hundred days after CA, Ulysses had reached a distance of 1.6 AU from Jupiter and an ecliptic latitude of -9°. The spacecraft spins around an axis which, along with the high-gain antenna, points towards Earth. The dust detector onboard has a 140° conical field-of-view (FOV), and is mounted almost at a right angle (85°) to the Ulysses spin axis. Radiant directions from which it can sense impacts therefore include the plane perpendicular to the spacecraft–Earth line. The rotation angle of the sensor axis at the time of a dust impact is measured from the ecliptic north direction. The spin-averaged sensitive area² of the dust detector to a mono-directional stream of dust grains is ≤ 0.02 m²; the maximum occurs when the centre of the detector FOV passes through the stream during spacecraft rotation.

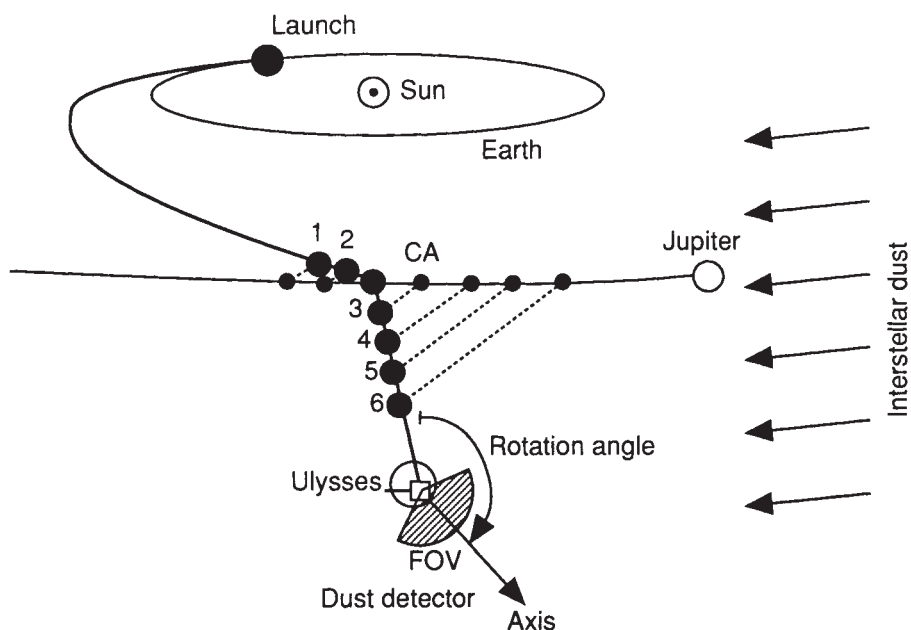
TABLE 1 Dust burst characteristics

Days from CA	-57.7	-32.1	31.4	59.8	86.9	117.4
Date (yr/d)	91/346	92/7	92/71	92/99	92/126	92/157
Duration (h)	4.7	6.0	25.0	43.4	19.8	16.3
Number of particles	3	4	124	7	4	4
Mass range ($\times 10^{-15}$ g)	3–6	0.1–7	1–90	2–9	5–20	3–4
Mean mass ($\times 10^{-15}$ g)	4	3	9	4	9	4
Speed range (km s ⁻¹)	28–37	27–56	28–44	28–44	20–37	28–37
Mean speed (km s ⁻¹)	31	37	42	33	29	30
Mean rotation angle	201°	211°	51°	54°	44°	32°
Distance to Sun (AU)	4.93	5.14	5.40	5.39	5.38	5.36
Distance to Jupiter (R_J)	995	562	553	1025	1480	1980

The time corresponds to the centre of the burst. Closest approach (CA) to Jupiter occurred on 92/39.5. The definition of stream particles and hence the number of members is somewhat arbitrary, but here it refers only to small (mass $\leq 5 \times 10^{-14}$ g) and collimated ($\pm 70^\circ$ from mean rotation angle) particles. Jupiter radius $R_J = 71,400$ km.

The Ulysses dust detector is a multi-coincidence impact ionization detector² with a sensitivity 10^5 times higher than any dust detector previously flown in the outer Solar System. Masses and impact speeds of dust particles are determined from the measured amplitudes and rise-times of the impact charge signals. We restrict our analysis here to reliably identified³ impact events (that is for small impact events, triple coincidence is required). The mass sensitivity threshold is 4×10^{-15} g at 20 km s⁻¹ and 6×10^{-16} g at 40 km s⁻¹ impact speed, as deduced from laboratory impact calibrations with carbon, silicate and iron dust particles⁴. The accuracy of the speed determination is a factor of two and that of the mass determination is a factor of 10 in the calibrated range⁵. From 8 days before closest approach (CA) to Jupiter until 2 days after, the instrument sensitivity was reduced by ground command by about a factor of two. For 17 hours each side of CA this sensitivity was further reduced by a factor of more than 10, for reasons of instrument safety. The trajectory of Ulysses and the geometry of dust detection is explained in Fig. 1.

The impact rate observed by Ulysses (Fig. 2) of big particles was low (~ 0.3 impacts per day) for most of the time, although a statistically significant peak of big particles did occur at the time of Jupiter encounter⁶. For most of 1991, when Ulysses was < 4 AU from the Sun, the impact rate of small particles was also low. Within a few months of Jupiter fly-by, however, six bursts



in the impact rate were observed, which occurred with a remarkable periodicity of 28 ± 3 days (Table 1). Although only three or four impacts in a single burst may seem debatable, the periodicity of their occurrence enormously increases their statistical significance. No periodic dust phenomena in interplanetary space have been known before for such small grains.

Figure 3 shows the direction that the sensor was pointing during impacts. Small particles ($\text{mass} \leq 5 \times 10^{-14}$ g) appear mostly in well collimated streams of short duration: a collimated stream, whose radiant passes through the detector field-of-view (FOV) once each spacecraft rotation, will be sensed over a range of a maximum of 140° . In Fig. 3b we show the same data as in Fig. 3a, only this time we have marked the larger particles according to their impact speed. No particles with impact speeds above 26 km s^{-1} (which represents the maximum possible vectorial sum of the Ulysses post-Jupiter heliocentric velocity of 8 km s^{-1} and the escape velocity of 18 km s^{-1} from the solar system at 5.4 AU) can move on bound orbits about the Sun. Therefore, all particles with impact speeds $> 26 \text{ km s}^{-1}$ move on hyperbolic trajectories through the Solar System. Even particles with lower impact speeds can be on hyperbolic orbits if they arrive from northerly directions or if radiation pressure reduces their solar attraction.

Jovian dust streams. In our opinion the following four characteristics of the dust streams, taken as a whole, can be explained only if we assume a jovian origin: (1) narrow, collimated streams must have a nearby source, otherwise they should be dispersed in space and time; (2) the streams are concentrated near Jupiter and the strongest stream was detected closest to it; (3) the first two streams before Jupiter CA approach Ulysses from directions almost opposite to the streams after CA. All streams, however, radiate from close to the line-of-sight direction to Jupiter. (4) The observed periodicity strongly suggests that all streams are derived from a single source and tends to rule out fortuitous cometary or asteroidal origins of individual streams.

The first three dust streams occurred when the plasma instrument⁷ and the magnetometer⁸ on Ulysses recorded 'corotating interaction regions' (ref. 9) in the solar wind and interplanetary magnetic field (IMF). These events were associated with intense magnetic fields (up to 5 nT) and high solar wind speeds (up to 600 km s^{-1}). Under these conditions the speed gained by the Lorentz force on a $0.1\text{-}\mu\text{m}$ -sized (or smaller) charged¹⁰ dust particle can be up to several kilometres per second per day. Particles in the first two streams arrived from about 50° south of the direction to Jupiter. The azimuthal component of the IMF at this time had maximum values in the direction opposite to planetary motion, which gave rise to a strong northward acceleration. The polarity and strength of the local IMF seem to be sufficient to bend the trajectories of the small stream

particles enough for them to be detected from the direction indicated.

Assuming that the observed streams originate from within the jovian system we still need to identify the source and ejection mechanism, and explain the periodicity. Any of the dusty regions (main ring, halo and the gossamer ring)^{11,12} could be the source of the observed particles. Also, the volcanoes on Io have been suggested^{13,14} as a source of submicrometre grains. Grains from any of these sources are exposed to the jovian magnetospheric plasma and will collect electrostatic charge. Their motions may then be dominated by electromagnetic forces that can overcome gravity and possibly lead to ejection. If particles originate from the dusty regions of Jupiter, one could argue for a sheet of continuously ejected dust similar in morphology to the interplanetary current sheet. The modulation of this dusty sheet could be due to a combination of the tilted nature of the jovian magnetic field and the solar wind. The modulation by the solar wind might manifest itself by Lorentz forces on the grains as they travel outside the magnetosphere—in this case the streams would correlate with the azimuthal component of the IMF—or also by the changing morphology of the jovian magnetosphere itself due to changes in the solar wind ram pressure. We note the similarity of the periods of dust streams with that of long-wave radio emissions from Jupiter, which were detected by the Voyager spacecraft and which are related to the solar wind magnetic sector structure¹⁵. If the source is Io, the dust sheet would probably reduce to rays or fans and there will be an additional modulation due to the orbital motion of Io¹⁶.

More modelling of the detailed dust emission mechanism and the subsequent accelerations needs to be done. Future measurements by the Galileo dust detector⁵, however, will provide new information on this new population of dust when Galileo reaches Jupiter late in 1995.

Interstellar dust. All planets and asteroids, and most short-period comets, as well as most interplanetary dust particles, orbit the Sun in the prograde sense (counterclockwise). With the spinning Ulysses spacecraft we are able to distinguish pro- and retrograde orbits. After Jupiter fly-by the spacecraft velocity component parallel to the ecliptic plane was small ($\sim 1.5 \text{ km s}^{-1}$) and hence dust particles in prograde orbits appear well separated in rotation angle (180° to 360°) from particles in retrograde (0° to 180°) motion. Before the encounter with Jupiter, this separation is less clear. Big particles ($\text{mass} > 5 \times 10^{-14}$ g) display a non-uniform distribution in rotation angle (Fig. 3): most impacts are compatible with retrograde motion. This conclusion was already indicated by the ratio of impact rates before and after Jupiter fly-by⁶.

The only class of objects in the Solar System which has high orbital inclinations and retrograde orbits are comets. Even long-period comets, which show a nearly random inclination distribution, do not match the observed dust particle distribution. Most of the dust released from these comets should be observable in the inner Solar System as well. Earlier measurements¹⁷ by Galileo and Ulysses show that significant amounts of high-inclination dust do not occur in the inner Solar System¹⁸.

A straightforward explanation for many of the retrograde orbits is that they are interstellar grains sweeping through the Solar System. This is because the upstream direction of interstellar dust (assumed to coincide with the flow of interstellar helium which has been directly measured by Ulysses¹⁹) is located at ecliptic longitude $l = 252^\circ$ and latitude $b = 2.5^\circ$, whereas Ulysses is currently at an almost right angle ($l = 157^\circ$) as seen from the Sun. Because of this configuration, interstellar dust particles, which directly reach the position of Ulysses, appear to have retrograde orbits (see Fig. 1).

The speed of interstellar grains should be high and comparable to the speed of interstellar gas, which is 26 km s^{-1} (ref. 19) outside the gravitational influence of the Sun. The speed of the particles should also be reflected in the apparent direction (rotation angle) from which these particles arrive at the detector.

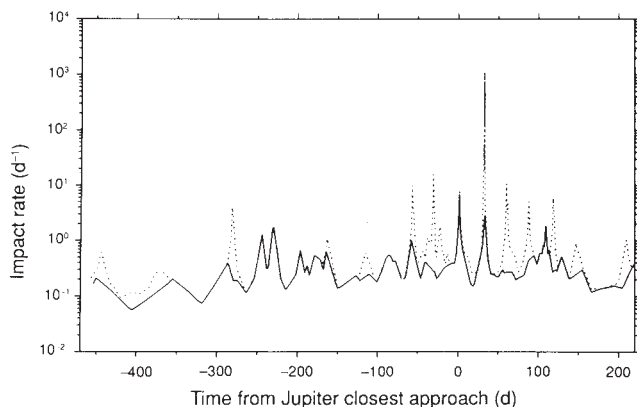


FIG. 2 Impact rate (from the Ulysses dust detector) from October 1990 to July 1992. Jupiter closest approach (CA) occurred on 8 February 1992. Solid line: impacts of big particles ($\text{mass} > 5 \times 10^{-14}$ g); broken line: all recorded impacts. Running average is over four impacts.

Because the spacecraft (apex) velocity vector changes by 80° in rotation angle at Jupiter (see Fig. 3) the relative impact velocities should shift accordingly. The mean rotation angle of slow ($<26 \text{ km s}^{-1}$) particles shifts by about 50° at Jupiter, whereas the shift for fast particles (excluding stream particles) is only 20° . These shifts are in agreement with the measured speeds and give credibility to these measurements.

The masses of the fast particles range from 10^{-15} to $5 \times 10^{-12} \text{ g}$, except for the time around Jupiter CA when even bigger particles were observed. The flux of those particles compatible with interstellar dust, both in rotation angle and speed, is $8 \times 10^{-5} \text{ m}^{-2} \text{ s}^{-1}$; if we take a mean particle mass to be $8 \times 10^{-13} \text{ g}$, the mass flux becomes $6 \times 10^{-17} \text{ g m}^{-2} \text{ s}^{-1}$. This value compares to $2 \times 10^{-17} \text{ g m}^{-2} \text{ s}^{-1}$ for an estimate² of interstellar dust in the

vicinity of the Solar System. This latter value is lower than the observed one, especially if one considers that the particles observed by Ulysses are bigger than the typical interstellar particles (mass $<10^{-14} \text{ g}$) which dominate the extinction of starlight.

McDonnell and Berg²⁰ estimated that at 1 AU from the Sun the upper limit flux of interstellar particles did not exceed $3 \times 10^{-5} \text{ m}^{-2} \text{ s}^{-1}$ for grains with a mass of 10^{-13} g . It has been suggested^{21,22} that both radiation pressure and electromagnetic interactions with the IMF may reduce the flux of interstellar grains in the inner Solar System. Ulysses dust observations indicate that in fact only the biggest interstellar particles are able to penetrate relatively deep into the Solar System (5 AU from the Sun) during the present magnetic field configuration²³. The probable identification of individual interstellar grains is of interest because it suggests that *in situ* analysis of such grains may be possible. Chemical analysis of these particles will be attempted in the future by the Cosmic Dust Analyzer on the Cassini spacecraft. □

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- Wenzel, P., Marsden, R. G., Page, D. E. & Smith, E. J. *Astr. Astrophys. Suppl.* **92**, 207–219 (1992).
- Grün, E. et al. *Astr. Astrophys. Suppl.* **92**, 411–424 (1992).
- Baguhl, M., Grün, E., Linkert, D., Linkert, G. & Siddique, N. in *Hypervelocity Impacts in Space* (ed. McDonnell, J. A. M.) 153–159 (Univ. of Kent, Canterbury, 1992).
- Göller, J. R. & Grün, E. *Planet. Space Sci.* **37**, 1197–1202 (1989).
- Grün, E. et al. *Space Sci. Rev.* **60**, 317–340 (1992).
- Grün, E. et al. *Science* **257**, 1550–1552 (1992).
- Bame, S. J. et al. *Astr. Astrophys. Suppl.* **92**, 237–266 (1992).
- Balogh, A. et al. *Astr. Astrophys. Suppl.* **92**, 221–236 (1992).
- Smith, E. J. & Wolfe, J. H. *Geophys. Res. Lett.* **3**, 137–140 (1976).
- Mukai, T. *Astr. Astrophys.* **99**, 1–7 (1981).
- Burns, J. A., Showalter, M. R., Cuzzi, J. N. & Pollack, J. B. *Icarus* **44**, 339–360 (1980).
- Showalter, M. R., Burns, J. A., Cuzzi, J. N. & Pollack, J. B. *Nature* **316**, 526–528 (1985).
- Johnson, T. V., Morfill, G. E. & Grün, E. *Geophys. Res. Lett.* **7**, 305–308 (1980).
- Morfill, G. E., Grün, E. & Johnson, T. V. *Planet. Space Sci.* **28**, 1101–1110 (1980).
- Zarka, P. & Genova, F. *Nature* **306**, 767–768 (1983).
- Horanyi, M., Morfill, G. E. & Grün, E. *Nature* (submitted).
- Grün, E. et al. *Geophys. Res. Lett.* **19**, 1311–1314 (1992).
- Divine, N. *J. geophys. Res.* (in the press).
- Witte, M., Rosenbauer, H., Banaszkiewicz, M. & Fahr, H. *Adv. Space Res.* (in the press).
- McDonnell, J. A. M. & Berg, O. E. in *Space Research Vol. 15*, 555–563 (Academic, Berlin, 1975).
- Morfill, G. E. & Grün, E. *Planet. Space Sci.* **27**, 1283–1292 (1979).
- Gustafson, B. A. S. & Misconi, N. Y. *Nature* **282**, 276–278 (1979).
- Morfill, G. E., Grün, E. & Leinert, C. in *The Sun and the Heliosphere in Three Dimensions* (ed. Marsden, R. G.) 455–474 (Reidel, Dordrecht, 1986).

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Time–distance helioseismology

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THE application of seismology to the study of the solar interior^{1,2} (helioseismology) has advanced almost solely by the prediction and measurement of the Sun's frequencies of free oscillation, or normal modes. Direct measurement of the travel times and distances of individual acoustic waves—the predominant approach in terrestrial seismology³—would appear to be more difficult in view of the number and stochastic nature of solar seismic sources. Here, however, we show that it is possible to extract time–distance information from temporal cross-correlations of the intensity fluctuations on the solar surface. This approach opens the way for seismic studies of local solar phenomena, such as subsurface inhomogeneities near sunspots, and should help to refine global models of the internal velocity stratification in the Sun.

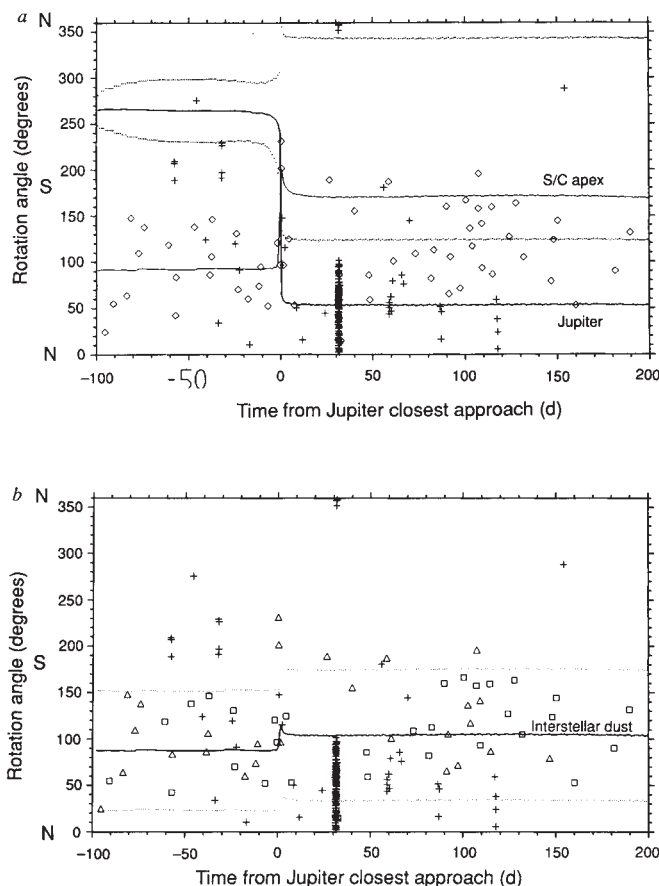


FIG. 3 Dust impacts versus spacecraft rotation angle and days from Jupiter closest approach (CA). Thirty-four out of 240 impacts are not shown because no correct rotation angle could be determined. The rotation angle is taken to be 0° when the dust sensor axis points closest to the north ecliptic pole. At rotation angle 90° the detector axis points parallel to the ecliptic plane in the direction of planetary motion, at 180° it points closest to the south ecliptic pole and so on. *a*, Impacts are marked according to their masses: diamonds: mass $>5 \times 10^{-14} \text{ g}$, crosses: mass $\leq 5 \times 10^{-14} \text{ g}$. The dotted lines on each side of the thick line labelled 'Jupiter' give the range of rotation angles when Jupiter is in the field-of-view (FOV) of the detector. The direction of spacecraft motion (S/C apex) is given by the thin line. Before Jupiter CA the detector pointed in the direction of spacecraft motion (S/C apex) at rotation angle $\sim 90^\circ$. After the fly-by the spacecraft went almost due south resulting in a new apex direction at rotation angle $\sim 170^\circ$. Impact directions of interplanetary dust particles, moving on low eccentricity and low inclination orbits, should follow this trend. *b*, Same data as in *a*, but marked according to the impact speeds. Small particles (crosses) all have impact speeds above 26 km s^{-1} . Impact speeds of bigger particles are identified by two different symbols: squares, $v \geq 26 \text{ km s}^{-1}$; triangles, $v < 26 \text{ km s}^{-1}$. The arrival direction of interstellar dust (assumed to coincide in direction and speed with interstellar gas¹⁹) is shown by the solid line, and the limits of detector FOV for interstellar grains are given by the dotted lines.

Seismology, the study of earthquakes and waves radiated by earthquakes and explosions, has been fundamental to our understanding of the Earth's interior structure. The observations that have contributed most to this understanding are wave travel times and distances, free oscillation (or normal mode) frequencies, and dispersion of surface waves⁴.

The Sun also experiences 'seismic disturbances' although, unlike the Earth where isolated large events are the main sources of seismic waves, solar seismic events are continually produced by a myriad of acoustic sources whose probable origin is the turbulent convection just beneath the solar surface^{5,6}. Because of the stochastic nature and location of these acoustic sources, studies of the solar interior by means of its seismic waves have so far concentrated on measurements of the Sun's standing wave (or normal mode) frequencies^{1,2}. The acoustic waves are confined within a resonant cavity which reflects waves downward near the surface and refracts them upward at a depth dependent on horizontal wavenumber k and frequency ω (refs 7, 8).

The essence of our new technique is to measure directly the times (t) taken by acoustic waves to travel from the solar surface to the bottom of their cavity and back, and also the distances (Δ), measured as an angle at the surface between the surface reflections. We demonstrate two ways of doing this. First we cross-correlate the wave observed at a point with the signal averaged over increasingly larger annuli (centred on that point) as a function of travel time. Second, we cross-correlate all points on the surface with all possible annuli as a function of travel time.

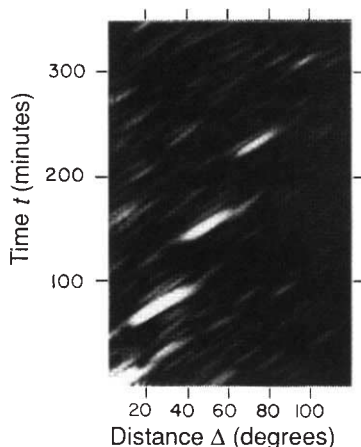
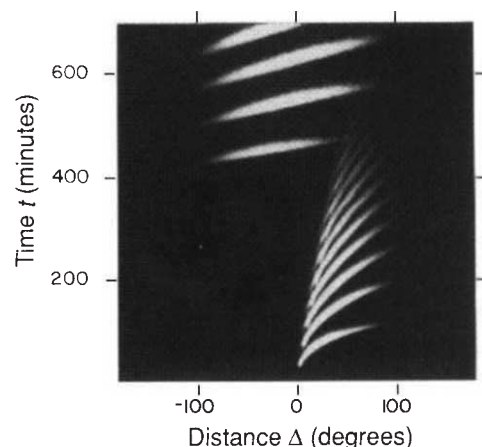


FIG. 2 The demodulated t - Δ autocorrelation derived from an ω - k power spectrum. The lowest curve represents one internal transit of the waves. The curves vertically above the first one correspond to the second, third and so on reflections from the surface. Different points along a curve correspond to waves penetrating to different depths. In addition to an instantaneous amplitude function, the demodulation procedure also yields an instantaneous phase function at each time t and distance Δ . The angular phase speed, w , at each point in the diagram can be calculated from the derivatives of this instantaneous phase function with respect to t and Δ . Waves of a given w are the points on which a line outward from the origin crosses the different curves. The width of the time-distance curves increases only slowly with transit number, so more accurate measurements of the time-distance relation can be made from the curves arising from multiple transits. The curves of reduced slope that cross $\Delta=0$ near $t=460$, 560 and so on are waves that are returning after travelling around the Sun. The waves that cross the Δ axis are those that undergo an integral number of skips on this circuit. The 'line profile' obtained by cutting across a single curve at fixed Δ as a function of t is the Fourier transform of the power in the frequency direction in the ω - k diagram. The inability to see the far side of the Sun causes the envelope of signal to be small near $\Delta \sim 180^\circ$. At longer travel times than those shown, waves with different phase speeds overlap, leading to interference of the time-distance curves. Quantitative work at large t will therefore require the isolation of waves with a small range of w .

Figure 1 shows our first measurements of t and Δ for acoustic waves that are trapped in a cavity $\sim 10^8$ m deep. These measurements were obtained from a reduction of 4,096 full-disk images of the Sun at 393 nm acquired at one-minute intervals from the geographic South Pole during November 1988⁹. The reduction procedure was as follows: (1) each image was decomposed into a set of spherical harmonic coefficients¹⁰ with $kR_\odot \leq 200$ (where $R_\odot$ is the solar radius); (2) the coefficients were transformed, using rotation matrices for spherical harmonics¹¹, to a co-ordinate system with a pole near the centre of the solar disk and rotating with the Sun at the mean photospheric rate; (3) the coefficients of circularly symmetric waves for each image were inverse transformed back to the spatial domain (the reason for only selecting circularly symmetric waves was to keep the reduction simple); (4) the resulting distance-time data were Fourier transformed to separate the waves moving towards and away from the origin; (5) the separated waves were filtered to isolate those with phase speeds at the solar surface of $w/2\pi \approx 40$ μ Hz (this isolation is possible because waves with a particular angular phase speed $w = \omega/kR_\odot$, all penetrate to the same depth⁷); (6) the filtered waves were inverse transformed back to the spatial domain; (7) the cross-correlation function¹² was calculated between the signal at an origin on the solar surface and the signal averaged over an annulus centred on the origin as a function of time and annulus radius; (8) strong modulation of the output signal, caused by the period of the waves that were admitted to the analysis, was removed (demodulated) using a two-dimensional analogue of the analytic signal analysis¹².

FIG. 1 A time-distance (t - Δ) diagram showing the demodulated temporal cross-correlations of circularly symmetric waves with $35 \mu\text{Hz} \leq w/2\pi \leq 45 \mu\text{Hz}$ at a pole, with the average signal in an annulus as a function of the annulus distance from the pole. Strong correlations from lower left to upper right represent one, two, three and four reflections of the waves travelling away from the pole. The decrease in intensity with increasing distance indicates progressive loss of wave energy.



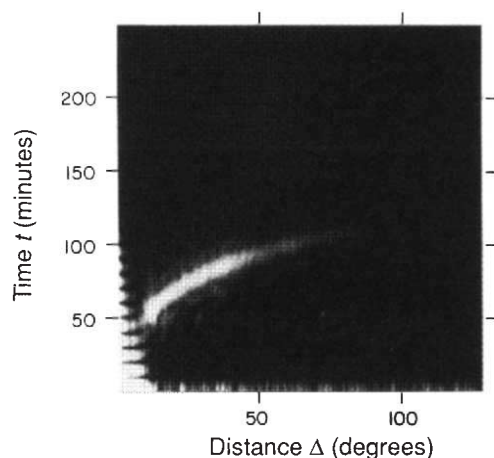


FIG. 3 The high-frequency time-distance diagram. Only frequencies $6.5 \text{ mHz} \leq \omega/2\pi \leq 8.3 \text{ mHz}$ are analysed. The acoustic cutoff frequency of the atmosphere is $\omega_{ac}/2\pi \sim 5.2 \text{ mHz}$. Only one curve is seen, implying that very little high-frequency wave energy is reflected downward near the solar surface. The noise along the axes is caused by a non-zero mean of the spectrum. From the background level, we estimate that the reflection of energy at the surface is $<2\%$ in this frequency range.

This method also works when the single point origin is replaced by any of the annuli acting as a source. This implies that the two-dimensional autocorrelation function¹² of the signal averaged over annuli will show a much better version of the result, because it effectively averages the correlation for all annuli of a given separation. It also reveals a connection with the ω - k power spectrum from a normal mode analysis: the autocorrelation function before demodulation is just the Fourier transform of the ω - k diagram¹³. So, in addition to the reduction technique used to generate Fig. 1, the wave travel times and distances can also be directly measured by Fourier transforming the ω - k diagram (obtained by a normal mode analysis) and demodulating the result. Unlike the first method, however, this latter method is unable to provide information on localized regions of the Sun. Figure 2 shows the demodulated autocorrelation function obtained from an ω - k diagram based on a spherical harmonic expansion of a 17-day series of solar oscillation observations⁹. The enhanced signal-to-noise ratio of the t - Δ diagram in Fig. 2, compared to that in Fig. 1, is due to: (1) the inclusion of waves with all azimuthal orders (corrected for effects of asphericity and solar rotation), into the reduction⁹ and (2) the use of the two-dimensional autocorrelation function instead of the one-dimensional analysis.

We note that the autocorrelation function derived from Fourier transforming an ω - k power spectrum based on a spherical harmonic expansion of the data, is only approximate. Consequently, the present analysis may have some quantitative defects related to this. A better analysis would include a three-dimensional cross-correlation of the data.

One immediate application of the t - Δ diagram is in the study of the properties of acoustic waves with frequencies ω greater than the acoustic cutoff frequency of the atmosphere (ω_{ac}). This is the wave frequency above which the wave travel time across a density scale height of the atmosphere is less than the period of the wave. In a classical analysis, waves with $\omega \gg \omega_{ac}$ are not reflected near the surface of the Sun, but travel out through the atmosphere and are lost to the system. Their normal mode spectrum is therefore expected to be continuous. Observations of these high frequency waves¹⁴, however, show modal behaviour in the ω - k diagram at high frequencies. One model¹⁵ to explain such observations asserts that this behaviour is due to an interference in the analysis procedure between a direct wave from a source with the wave that has travelled to the bottom of the cavity and returned to the surface. Here the high-frequency waves carry information on the position and extent of the source. An alternative model¹⁶ claims that partial reflection of the waves in the photosphere (or at the transition region between the chromosphere and corona) causes a resonance. If this is true, then the observed high-frequency modal structure contains information on the mean structure of the solar atmosphere. The importance of distinguishing between these two models has been discussed by Brown¹⁷.

If reflections are indeed significant, we would expect to see multiple curves in the t - Δ diagram for waves with $\omega \gg \omega_{ac}$. If they are not, there should only be a single curve, corresponding to the correlation between a direct wave source and the downward wave that has been refracted and has returned to the surface. The reduction leading to Fig. 2 was therefore repeated with additional filtering in frequency to admit only waves with $\omega/2\pi > 6.5 \text{ mHz}$. The results are shown in Fig. 3 and demonstrate that any reflection of these waves must be weak, favouring the first model.

As for seismology, the ability to measure travel times and distances will be important for helioseismology. The direct measurement of wave travel times and distances will be of great benefit to the study of local problems, such as inhomogeneities near sunspots. Similarly, maps of travel times and distances could be determined locally to study subsurface inhomogeneities. Subsurface flows that cause advection of the waves will directly affect the travel times and distances of the waves and will be visible through this signature.

Use of the t - Δ diagram will also benefit measurement of the global properties of the Sun. The variables t and Δ are closer than ω and k to the functions needed to determine the internal sound speed stratification. The lifetime of short-wavelength waves will be given by the decrease of amplitude with position along a line from the origin, corrected for the natural decrease caused by our observing window. This measurement is difficult in the ω - k diagram because the mode line profiles used to measure the lifetimes are unresolved (owing to our inability to see the far side of the Sun). In addition, there is always the possibility that some new effect, not easily visible in the ω - k diagram, will be readily apparent in a t - Δ diagram. $\square$

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- Hill, F., Deubner, F.-L. & Isaak, G. in *Solar Interior and Atmosphere* (eds Cox, A. N., Livingston, W. C. & Matthews, M. S.) 329-400 (Univ. Arizona Press, Tucson, 1991).
- Christensen-Dalsgaard, J. & Berthomieu, G. in *Solar Interior and Atmosphere* (eds Cox, A. N., Livingston, W. C. & Matthews, M. S.) 401-478 (Univ. Arizona Press, Tucson, 1991).
- Jeffreys, H. *The Earth*, 4th edn (Cambridge Univ. Press, 1959).
- Gubbins, D. *Seismology and Plate Tectonics* (Cambridge Univ. Press, 1990).
- Kumar, P. & Goldreich, P. *Astrophys. J.* **342**, 558-575 (1989).
- Osaki, Y. in *Progress of Seismology of the Sun and Stars* (eds Osaki, T. & Shibahashi, H.) 75-86 (Lecture Notes in Physics 367, Springer, Berlin, 1990).
- Ulrich, R. K. *Astrophys. J.* **162**, 993-1002 (1970).
- Leibacher, J. W. & Stein, R. F. *Astrophys. Lett.* **7**, 191-192 (1971).
- Duvall, T. L. Jr, Jefferies, S. M., Harvey, J. W., Osaki, Y. & Pomerantz, M. A. *Astrophys. J.* (in the press).
- Brown, T. M. *Nature* **317**, 591-594 (1985).
- Christensen-Dalsgaard, J. *Solar Seismology from Space* 219-253 (Jet Propulsion Laboratory Publication 84-84, Pasadena, 1984).
- Bracewell, R. *The Fourier Transform and its Applications* (McGraw-Hill, New York, 1965).
- Jennison, R. C. *Fourier Transforms and Convolutions for the Experimentalist* (Pergamon, New York, 1961).
- Duvall, T. L. Jr, Harvey, J. W., Jefferies, S. M. & Pomerantz, M. A. *Astrophys. J.* **373**, 308-316 (1991).
- Kumar, P. & Lu, E. *Astrophys. J.* **375**, L35-L39 (1991).
- Balmforth, N. J. & Gough, D. *Astrophys. J.* **362**, 256-266 (1990).
- Brown, T. M. *Astrophys. J.* **371**, 396-401 (1991).

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Increased transition temperature in superconducting $\text{Na}_2\text{CsC}_{60}$ by intercalation of ammonia

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A WIDE variety of alkali-metal fullerides (A_xC_{60}) have been prepared, with stoichiometries varying between $1 \leq x \leq 11$ (refs 1, 2). Most of the compounds with $x = 3$ are superconductors with unusually high transition temperatures (T_c) which increase with the size of the face-centred cubic unit cell³. The increase in T_c on lattice expansion is attributed to a decrease in the conduction band width, and therefore one ultimately expects a saturation or downturn of T_c followed by a metal-insulator transition. We have sought to explore this regime by expanding the A_3C_{60} structure through intercalation of neutral molecules capable of solvating the A^+ ions. Here we report that the reaction of ammonia with $\text{Na}_2\text{CsC}_{60}$ ($T_c = 10.5$ K; lattice constant $a = 14.132$ Å) produces the compound $(\text{NH}_3)_4\text{Na}_2\text{CsC}_{60}$, which has an expanded unit cell ($a = 14.473$ Å) and an increased T_c of 29.6 K. The structure contains the $\text{Na}(\text{NH}_3)_4^+$ cation on the octahedral site.

The A^+ ions in cubic A_3C_{60} occupy octahedral and tetrahedral interstices between C_{60} molecules. The large difference between the radius, r , of the octahedral and the tetrahedral sites in f.c.c. A_3C_{60} ($r_{\text{oct}} = 0.5a - r_{\text{C}_{60}}$, $r_{\text{tet}} = 0.434a - r_{\text{C}_{60}}$, $r_{\text{C}_{60}} \approx 5.0$ Å) accounts for cation ordering when the A^+ ion is large (as in $\text{Na}_2\text{CsC}_{60}$). The octahedral site is larger than any atomic cation, leading to large crystallographic thermal parameters for octahedral A^+ ions⁴ and possible displacements from the centre of the sites⁵. The recognition that cationic calcium⁶ and sodium^{7,8} clusters could occupy the octahedral site suggests that other small molecular cations can occupy this site, giving the opportunity to access larger unit cells while maintaining the desired C_{60}^{3-} oxidation state. The smallest molecular cation expected to be chemically compatible with C_{60}^{3-} is $(\text{CH}_3)_4\text{N}^+$. After several unsuccessful attempts to incorporate this ion by ion exchange

TABLE 1 Structural parameters and nearest-neighbour bond distances of $(\text{NH}_3)_4\text{Na}_2\text{CsC}_{60}$

	Site	x	y	z	N	B
C(1)	48(h)	0.0	0.04914	0.2383	1	1.7(3)
C(2)	96(i)	0.2080	0.07943	0.09807	1	1.7(3)
C(3)	96(i)	0.1775	0.1587	0.04914	1	1.7(3)
Na(1)	8(c)	0.25	0.25	0.25	0.5	5.0(3)
Na(2)	4(b)	0.5	0.5	0.5	1.0	1.9(6)
Cs(1)	8(c)	0.25	0.25	0.25	0.5	5.0(3)
N(1)	32(f)	0.4	0.4	0.4	0.51(2)	3.0(7)
H(1)	96(i)	0.3584	0.3584	0.4394	0.51(2)	3.0(7)
Nearest-neighbour interatomic distances (Å)						
N-Na(o)	N-N	N-Cs(t)	N-C	N-H	Cs(t)-C	H-C
2.5	2.89	3.76	2.81	1.02	3.37	2.29

* Space group $Fm\bar{3}$, $a = 14.473$ Å.

and direct reaction with $(\text{CH}_3)_4\text{NBH}_4$, we turned to the possibility of creating complexed cations by addition of molecular NH_3 to preformed A_3C_{60} .

Our initial studies of the reactions of A_3C_{60} with NH_3 gas suggest that a rich variety of ternary intercalation compounds involving alkali metals and NH_3 can be formed with structures and stoichiometries that depend on the alkali metal(s), ammonia pressure and reaction temperature. The general trends observed so far are consistent with known NH_3 chemistry: coordination strength decreases with increasing A^+ size. Under similar conditions of ~ 0.5 atm NH_3 and a temperature of 100°C , Rb_3C_{60} failed to react, K_3C_{60} yields $(\text{NH}_3)_3\text{K}_3\text{C}_{60}$, and $\text{Na}_2\text{AC}_{60}$ ($\text{A} = \text{Rb}, \text{Cs}$) affords $(\text{NH}_3)_4\text{Na}_2\text{AC}_{60}$. Here we discuss mainly the experimental conditions, and the results of ammoniation of $\text{Na}_2\text{CsC}_{60}$. $(\text{NH}_3)_3\text{K}_3\text{C}_{60}$ will be described elsewhere (M.J.R. *et al.*, manuscript in preparation).

The $\text{Na}_2\text{CsC}_{60}$ sample used in this study was characterized previously⁹ as an f.c.c. superconductor with $a = 14.132$ Å, $T_c = 10.5$, with a diamagnetic shielding fraction of 8%. The sample was exposed to 380 torr NH_3 gas distilled from a sodium/liquid-ammonia solution through a vacuum manifold. A substantial pressure drop was observed within a few minutes. After 1–2 days at room temperature, the pyrex reaction tube was sealed under the partial NH_3 pressure and annealed at 100°C for one day. A weight uptake measured on a similarly treated $\text{Na}_2\text{RbC}_{60}$ sample corresponds to addition of four moles of NH_3 per mole of sample. Once reacted, the tubes were opened in a helium glove box and the product was stable to NH_3 loss in that environment.

The powder X-ray pattern of $\text{Na}_2\text{CsC}_{60}$ exposed to NH_3 gas is shown in Fig. 1. All the reflections, except the broad feature marked at about $19.2^\circ 2\theta$, can be indexed on the basis of an f.c.c. unit cell with a lattice parameter of $14.473(2)$ Å. This is a substantial expansion from the original unit-cell parameter of 14.132 Å. The origin of the broad peak at about 19.2° is not clear, but it is not associated with any known Na or Cs oxide, amide or hydroxide. Low-temperature powder X-ray diffraction studies showed that the f.c.c. structure persists down to 20 K.

Measurement of a.c. and d.c. magnetic susceptibilities indicate that ammoniated $\text{Na}_2\text{CsC}_{60}$ is superconducting with an onset temperature of 29.6 K, a nearly threefold increase over that of the unexpanded host. The d.c. susceptibilities before and after the reaction are shown in Fig. 2. The diamagnetic shielding fraction of the ammoniated sample is about 6%, comparable with that of the starting material, and consistent with superconductivity in a small-grained sample (≤ 1 µm). The observed T_c is in line with the value expected based on the nearly linear correlation of T_c with a in the A_3C_{60} compounds^{3,10}. After a few minutes of gentle (but uncontrolled) heating under dynamic vacuum, the T_c of the ammoniated compound returned to that of the unexposed $\text{Na}_2\text{CsC}_{60}$ (10.5 K) and powder X-ray diffraction showed only $\text{Na}_2\text{CsC}_{60}$. These observations show that the

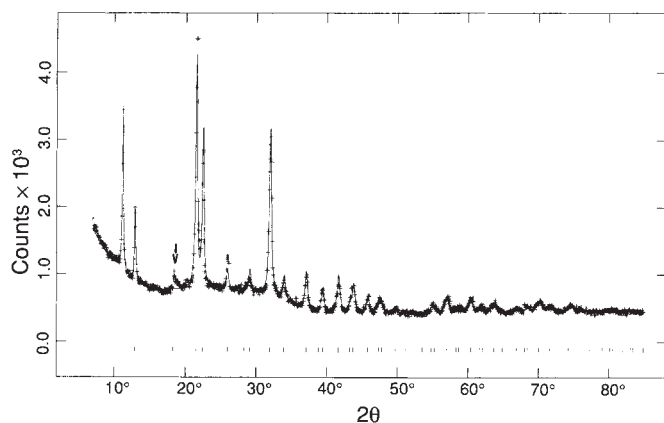


FIG. 1 Synchrotron X-ray powder diffraction pattern of $(\text{NH}_3)_4\text{Na}_2\text{CsC}_{60}$ sample contained in a sealed glass X-ray capillary. Data were collected at the National Synchrotron Light Source beamline X16B at 1.628 -Å wavelength. The crosses are experimental data and the solid line is a fit obtained from the Rietveld refinement with space group $Fm\bar{3}$ ($a = 14.473(2)$ Å, $wR_p = 7.9\%$ and $R_p = 5.7\%$).

ammonia intercalation is thermally reversible, and rule out the possibility of amide formation in the ammoniated structure. An early measurement, carried out when we were unaware of the ease of thermal reversibility, showed no superconductivity in $(\text{NH}_3)_4\text{Na}_2\text{RbC}_{60}$, although a T_c of about 31 K was expected on the basis of the expanded 14.509-Å f.c.c. lattice constant. It is likely that ammonia was driven out by heating under dynamic vacuum when the pyrex tube was sealed for susceptibility measurements. The thermal reversibility of ammonia intercalation has been documented in the well-studied ternary alkali-ammonia-graphite intercalation compounds, where the reversibility increases with increasing alkali ion size¹¹.

We have modelled the crystal structure of this new compound and carried out Rietveld refinement. Preliminary X-ray intensity calculations were carried out by varying the electron densities on the tetrahedral and octahedral sites using the LAZY-PULVERIX program (K. Yvon, W. Jeitschko and E. Parthe) and a spherical shell for the C_{60} X-ray form factor¹². The observed powder X-ray pattern of the ammoniated sample can be reasonably reproduced with 33 electrons on the tetrahedral sites and 22 electrons on the octahedral sites. Thus the electron density on the tetrahedral site has risen dramatically over that of $\text{Na}_2\text{CsC}_{60}$ (enough to give $\text{Na}(\text{NH}_3)_2$) with a decrease at the octahedral site. Examination of the sizes of the interstitial sites,

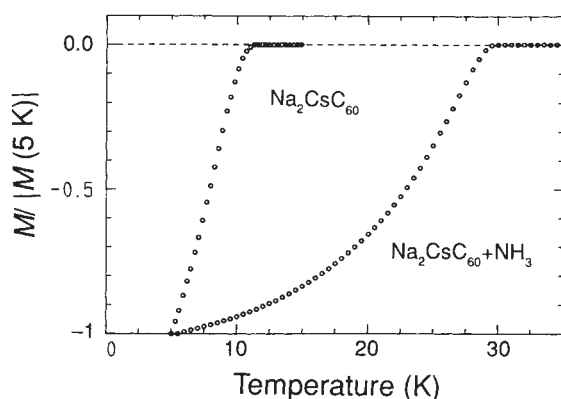


FIG. 2 Normalized d.c. magnetic susceptibilities of $\text{Na}_2\text{CsC}_{60}$ and $(\text{NH}_3)_4\text{Na}_2\text{CsC}_{60}$, measured in a field of 2.5 Oe.

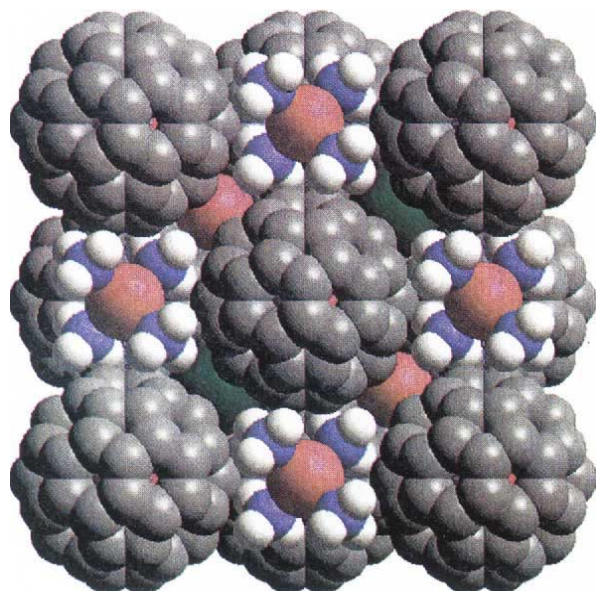


FIG. 3 A model of $(\text{NH}_3)_4\text{Na}_2\text{CsC}_{60}$ with ordered $\text{Na}(\text{NH}_3)_4$ tetrahedra. The C, Na, Cs, N and H atoms are represented by grey, red, green, blue and white spheres respectively.

however, indicates that the tetrahedral void is too small to accommodate the $\text{Na}(\text{NH}_3)_2$ complex. Therefore, we have considered a model in which Na and 4 NH_3 occupy the octahedral interstice and the remaining Na and Cs occupy tetrahedral sites.

A profile intensity calculation was carried out in space group $Fm\bar{3}$ for a model having Na and Cs disordered on equivalent tetrahedral sites (8(c) $(\frac{1}{4}, \frac{1}{4}, \frac{1}{4})$) and the remaining Na on the octahedral site (4(b) $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$). Nitrogen atoms are placed at the 32(f) (x, x, x) positions on the body diagonal of the cubic cell. In the initial refinement, the N-Na(o) distance was constrained at 2.5 Å, a typical distance found in NaX-NH_3 ($\text{X} = \text{Cl}, \text{Br}$)¹³. The occupancies of the Na(o), Na(t), and Cs(t) ions were fixed at 1, 0.5 and 0.5 respectively to conserve the initial stoichiometry. Hydrogens were ignored. With the Na-N distance fixed, the only variable positional parameters are those of the carbon atoms on the C_{60} . Previous X-ray refinements of A_xC_{60} have shown that intercalation of foreign species into the C_{60} lattice has only a minor effect on the C-C bonds in the C_{60} molecule¹⁴. We therefore allowed only radial dilation of the molecules. Refinement of the occupancy of N and the thermal amplitudes of all the elements present converged rapidly to a reduced χ^2 of 1.7, the respective profile intensity R factors of $wR_p = 7.9\%$ and $R_p = 5.8\%$, and the nitrogen occupancy of 0.62. An unconstrained refinement allowing the Na(o)-N bond distance, the N occupancy and the thermal factors to vary at the same time showed that these parameters are strongly correlated; therefore, the Na(o)-N bond distance was constrained in later refinements. As the three electrons from the hydrogen atoms constitute a significant fraction of the total electron counts in the NH_3 molecule, the hydrogens were then incorporated into the model and placed at the 96(i) general site, with coordinates fixed by constraining the H-N distance and the H-N-H bond angle and fixed rotation angle. The occupancy of N converged to 0.51 without significant change of the R factors in a refinement with the position of the NH_3 molecule fixed. This N occupancy suggests the presence of $\text{Na}(\text{NH}_3)_4$ tetrahedra in two disordered orientations, similar to the model proposed for Na_4 cluster in Na_6C_{60} (ref. 7). The structural parameters obtained from the refinement are given in Table 1.

We also considered two alternative models allowing either merohedral twinning of the C_{60} molecules, as reported for K_3C_{60} (ref. 4), or ordering of the tetrahedral alkali metal ions and the ammonia. Merohedral twinning gave an equivalent fit to the $Fm\bar{3}$ model. Ordering of the tetrahedral ions and NH_3 in space group $F23$ such that NH_3 is adjacent to either tetrahedral Na or Cs (with ordered C_{60}) was also considered. The arrangement with the NH_3 molecule bridging a pair of Na ions is enticing from the chemical point of view, but neither of these ordered models reproduces the observed intensities.

The $(\text{NH}_3)_4\text{Na}_2\text{CsC}_{60}$ f.c.c. unit cell is illustrated in Fig. 3 with ordered $\text{Na}(\text{NH}_3)_4$ tetrahedra for ease of viewing. A notable feature of this structure is the movement of the Cs ion from the octahedral site in $\text{Na}_2\text{CsC}_{60}$ to the tetrahedral site in this structure. If considered as a spherical ion, $\text{Na}(\text{NH}_3)_4^+$ has a radius of ~ 2.9 Å, much larger than the expected radius of the octahedral site of ~ 2.2 Å, but the tetrahedral geometry of the ion allows a favourable directional nesting of the NH_3 s in the trigonal hole between three C_{60} molecules. Similar nesting would favour cubic, eight-coordinate ions, but would mitigate against octahedral, six-coordinate ions. Size considerations alone do not seem to preclude the possibility of forming $\text{Na}(\text{NH}_3)_8^+$.

Although here we have focused on the sequential intercalation of alkali metals followed by ammonia, it should be possible to adapt the approach to intercalation of molecular cations and to synthesis directly from liquid ammonia or other solvents. In particular, because small, highly charged ions coordinate most strongly to ammonia, the use of trivalent rare-earth metals to form $\text{M}(\text{NH}_3)_4\text{C}_{60}$ or $\text{M}(\text{NH}_3)_6\text{C}_{60}$ is an attractive target for future studies. The ability to increase the cell size beyond that

available solely with atomic cations suggests that materials with unit cells large enough to identify the maximum possible T_c , and the expected metal-insulator transition beyond this, may be accessible synthetically. $\square$

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1. *Acc. Chem. Res.* **25**, 3 (eds McLafferty, F. W. & Smalley, R. E.) (Am. chem. Soc., 1992).
2. *J. Phys. Chem. Solids* **53** (eds Fischer, J. E. & Cox, D. E.) (Pergamon, Oxford, 1992).
3. Fleming, R. M. *et al. Nature* **352**, 787–788 (1991).
4. Stephens, P. W. *et al. Nature* **351**, 632–634 (1991).
5. Walstedt, R. E., Murphy, D. W. & Rosseinsky, M. J. *Nature* (in the press).
6. Kortan, A. R. *et al. Nature* **355**, 529–532 (1992).
7. Rosseinsky, M. J. *et al. Nature* **358**, 416–418 (1992).
8. Yildirim, T. *et al. Nature* **380**, 568–571 (1992).
9. Murphy, D. W. *et al. J. Phys. Chem. Solids* **53**, 1321–1332 (1992).
10. Zhou, O. *et al. Science* **255**, 833–835 (1992).
11. Setton, R. in *Graphite Intercalation Compounds I* (eds Zabel, H. & Solin, S. A.) 305 (Springer-Verlag, Berlin, 1990).
12. Heiney, P. A. *et al. Phys. Rev. Lett.* **66**, 2911–2914 (1991).
13. Olovsson, I. *Acta Cryst.* **18**, 879–889 (1965).
14. Zhou, O. & Cox, D. E. *J. Phys. Chem. Solids* **53**, 1373–1390 (1992).

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Computer simulation of hydrogen embrittlement in metals

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IT has been long known that hydrogen can substantially reduce the mechanical stability of transition metals under tensile stress^{1–3}. This phenomenon of 'hydrogen embrittlement' has important consequences for the safety of fusion reactors and for space technology; but there remains considerable uncertainty about its microscopic origin^{2,3}. Here we report the results of a study of fracture of hydrogen-loaded palladium under tensile stress which uses Parrinello–Rahman molecular dynamics based on a many-body alloy hamiltonian. A rather unexpected result is that the apparent hydrogen embrittlement results from a local enhancement of ductility in hydrogen-saturated regions of the metal which causes a reduction of the critical tensile stress at which failure occurs.

One of the oldest proposed mechanisms of hydrogen embrittlement is the 'decohesion mechanism' which associates this effect with a decreased metal bond strength in the presence of hydrogen^{4–7}. The 'hydrogen-related phase change mechanism' has been suggested as the origin of hydrogen embrittlement in those systems where a brittle hydride phase is stabilized by the presence of hydrogen and the crack-tip stress fields^{8,9}. The 'hydrogen-enhanced local plasticity' (HELP) mechanism postulates a ductility enhancement at crack tips which facilitates the initiation and propagation of fracture^{10–12}.

We elected to study the effect of hydrogen on the mechanical stability of Pd because this system shows a large variety of interesting effects when exposed to hydrogen, such as hydride formation and surface reconstruction, and has been studied extensively by *ab initio* techniques^{13–15}. To understand the microscopic processes associated with hydrogen-assisted crack formation, we used a molecular dynamics (MD) calculation¹⁶. This technique describes the evolution of the system in time by direct numerical solution of the equations of motion for individual atoms. We used the Parrinello–Rahman MD method^{17–19} to study specifically the elastic response (and mechanical stability) of bulk Pd to uniaxial tensile stress at different temperatures and concentrations of hydrogen. This approach provides the

framework for treating a canonical ensemble, exposed to an arbitrary stress field at a fixed temperature, by introducing 10 extra degrees of freedom for the coupling of the system to an external heat bath and a corresponding pressure reservoir. Specifically, this method can determine the deformations of the unit cell which occur during the fracture process, in response to an anisotropic stress field.

A precise description of atomic interactions is of critical importance to a successful description of the fracture process. In single-component systems with a close-packed structure, the embedded-atom method (EAM)^{20,21} has proved to be quite successful in describing structural and dynamical properties^{22,23}. Recently we developed a model many-body alloy (MBA) potential²⁴ based on *ab initio* calculations for alloy systems, and used this potential successfully to study the structure and dynamics of bulk Pd, clean and hydrogen-covered Pd surfaces at zero temperature²⁴ and the melting transition of Pd (ref. 25). The main advantage of the MBA over the EAM hamiltonian in alloys is that the MBA distinguishes neighbouring sites by both the atom type and the atomic charge density. It also does not assume local charge neutrality.

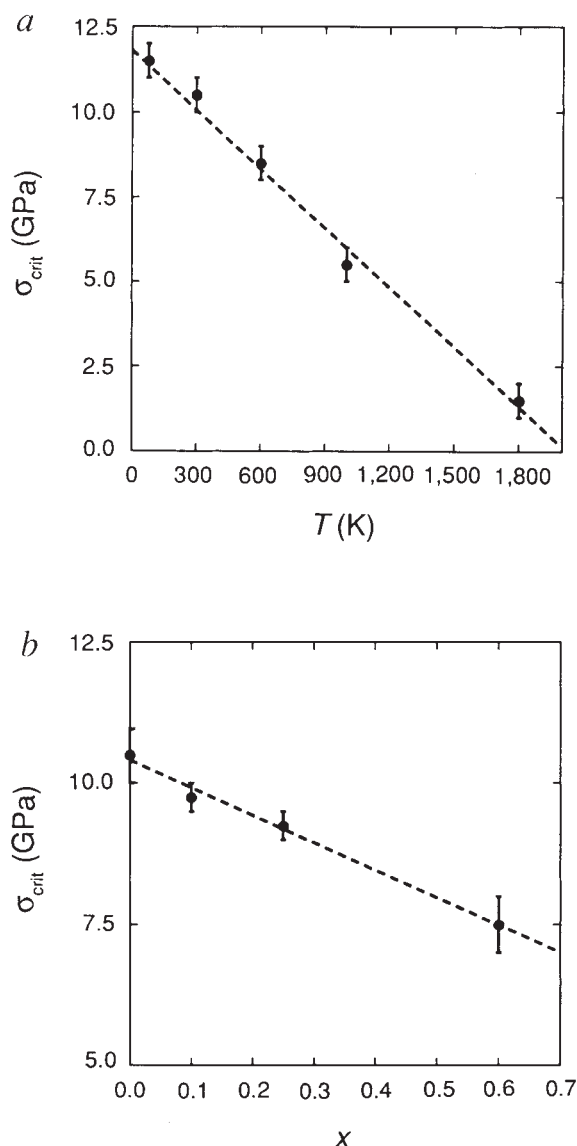


FIG. 1 Calculated critical tensile stress σ_{crit} for (a) hydrogen-free bulk Pd at different temperatures and (b) PdH_x at different hydrogen concentrations x . The dashed lines serve as guides to the eye.

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The parameters for the MBA hamiltonian appropriate for the Pd-H systems have been given in ref. 24. The dynamics of the system is obtained by integrating the equations of motion, which result from the Parrinello-Rahman lagrangian¹⁷⁻¹⁹. In a typical MD simulation of the bulk crystal, we consider a periodic arrangement of unit cells (with originally cubic shape) containing 500 Pd atoms. The effect of hydrogen concentration on the mechanical stability of PdH_x is investigated at four hydrogen concentrations, $x = 0.0, 0.1, 0.25$ and 0.6 . Initially, we let the system equilibrate at zero external pressure and a specific temperature. Then, we apply a uniaxial tensile stress σ to the system, which we gradually increase in small steps. Following each pressure increase, we let the system equilibrate. The critical tensile stress for fracture (σ_{crit}) is reached when the length of the unit cell along the direction of the tensile stress increases dramatically. This elongation is typically associated with the development of a large crack which makes the system unstable. At this point, the shape of the unit cell is frozen in and we monitor the time evolution of the crack. This leads to information on the mechanical failure process at a microscopic level.

We first address the effect of temperature on the mechanical failure in bulk Pd. In Fig. 1a we show the critical tensile stress σ_{crit} as a function of temperature T . Our results indicate a rapid linear decrease of σ_{crit} with increasing temperature. Note that the extrapolation of our data yields the reasonable result that the critical tensile stress vanishes near the calculated²⁵ melting point $T_m = 2,050$ K (which in turn is close to the experimental²⁶ value of 1,827 K). We expect our simulation to give an upper limit (rather than a realistic estimate) to the observed tensile stress in real materials, due to our use of an initially defect-free lattice and relatively large strain rate, which is reminiscent of a shock wave.

Next, we determine the effect of hydrogen concentration (x in PdH_x) on σ_{crit} . Figure 1b shows a linear decrease of the critical tensile stress with increasing hydrogen concentration. This behaviour is very similar to the dependency of σ_{crit} on temperature, as discussed above.

The similarity between Fig. 1a and b implies that presence of hydrogen may have a similar effect on σ_{crit} as a temperature increase. This behaviour may be due to a plasticity enhancement which eventually should lead to melting. To investigate whether the presence of hydrogen may cause local pre-melting of the system, we calculate the Pd-Pd pair correlation function $g(r)$ at the point of fracture for Pd at different temperatures and concentrations of hydrogen. Our results are shown in Fig. 2. The pair correlation function for pure Pd at 300 K (Fig. 2a) shows only weak vibrational broadening of the 'Bragg peaks' (associated with a crystalline solid) with visible structure up to very large values of r . As the temperature rises to 1,000 K (Fig. 2b), the nearest-neighbour peak in $g(r)$ still remains sharp. At large r , however, $g(r)$ gradually loses structure and approaches unity. This behaviour, together with a second split peak at $r \approx 5$ Å, indicates an amorphous structure, which has been formed under critical levels of tensile stress. Because melting is a typical prerequisite for glass formation, we conclude that stress-

induced pre-melting occurs in our system at a temperature well below the bulk melting point²⁶ ($T_m = 1,827$ K).

A striking result is the similarity between the Pd-Pd pair correlation functions of pure Pd at 1,000 K and that of PdH_{0.25} at 300 K (Fig. 2b and c respectively). This indicates that the atomic structures of bulk Pd and PdH_x at the point of fracture are very similar and may be amorphous. We conclude that hydrogen loading and an increase of temperature have a similar effect on the formation of cracks and subsequent mechanical failure. A possible cause of both these effects could be stress-induced pre-melting of the system.

The easiest way to verify this is to inspect the motion of the individual atoms in Pd and PdH_x at different temperatures during the fracture process. We have followed the atomic motion under tensile stress in a computer-animated movie. Characteristic atomic arrangements in a system subject to tensile stress along the horizontal direction are presented in Fig. 3, depicting three adjacent layers in bulk Pd (out of ten layers in the MD unit cell).

At 300 K, the atomic structure of pure Pd at a moment preceding the fracture, shown in Fig. 3a, is very regular and contains no explicit indication of where a crack might start forming. Upon applying tensile stress, we first observe an increased lattice spacing in the stress direction, and finally a disintegration of the system into isolated monolayers at σ_{crit} . These monolayers subsequently recombine to give thicker slabs with a very regular and smooth surface (see Fig. 3d). No significant atomic diffusion is observed in the crack area, indicating a brittle fracture.

At the higher temperature of 1,000 K, Pd shows increased atomic disorder (Fig. 3b). Thermal fluctuations induce defects which are potential seeds of microcracks under tensile stress. On the microscopic scale, fracture proceeds in a ductile manner, and occurs much faster than at room temperature. At the moment following the fracture, (Fig. 3e), we find a large atomic diffusion along the crack surface, indicating melting well below the bulk melting point. This diffusion smooths the originally rough crack surface, and leads to the amorphous structure discussed above.

The most important part of our study is the crack formation in PdH_{0.25} at 300 K, shown before and after the fracture in Fig. 3c and 3f, respectively. Before the point of fracture, we observe a large number of defects especially in regions saturated by hydrogen. These defects act as seeds for crack formation (see the arrow in Fig. 3c). Because hydrogen atoms are most stable in an expanded lattice²⁷, the strained lattice at the crack tip facilitates local accumulation of hydrogen. As discussed below, presence of hydrogen enhances ductility locally. This is associated with a local pre-melting, which in turn accelerates the crack propagation. Our computer animation shows that hydrogen strongly enhances the diffusion of Pd atoms near the crack surface, leading to a faster healing of a formed crack. As we see in the 'snapshot' in Fig. 3f of the geometry following the fracture, the presence of hydrogen leads to a microscopically plastic behaviour and a ductile fracture. This interpretation of the processes is obvious from a comparison of hydrogen-free

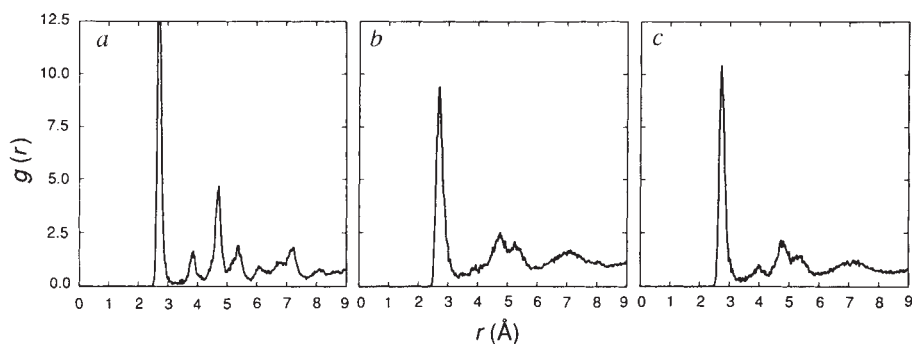


FIG. 2 The Pd-Pd pair correlation function $g(r)$ at the point of fracture for (a) bulk Pd at 300 K, (b) bulk Pd at 1,000 K, and (c) bulk PdH_{0.25} at 300 K.

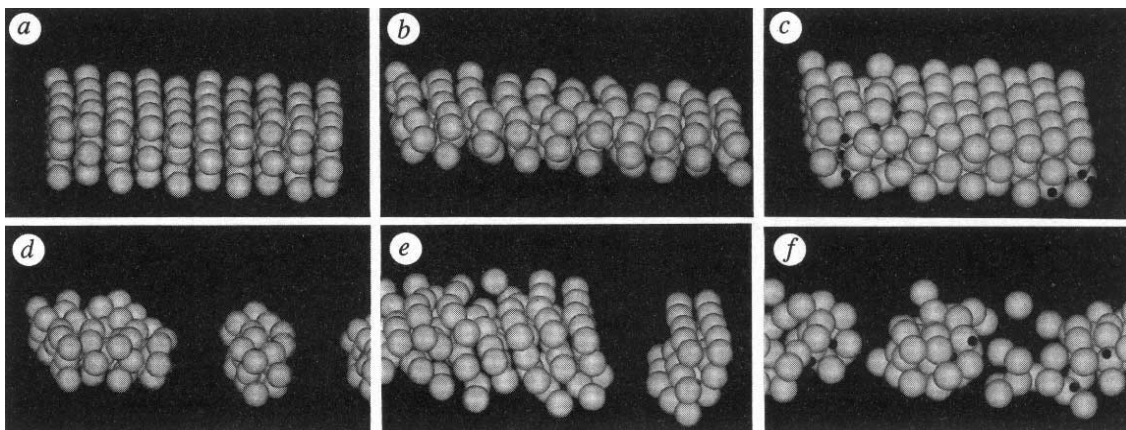


FIG. 3 Momentary atomic arrangement in three adjacent layers of the bulk metal before the point of fracture for (a) Pd at 300 K, (b) Pd at 1,000 K, and (c) PdH_{0.25} at 300 K. The corresponding geometries after fracture are

given in d, e and f. The Pd atoms are shown as white spheres with a radius of 1.3 Å and hydrogen atoms are given by black spheres with a radius of 0.4 Å.

bulk Pd (Fig. 3d) and bulk PdH_{0.25} (Fig. 3f) at the same temperature $T = 300$ K, at the moment following fracture.

We can explain the microscopic origin of the local hydrogen-induced ductility enhancement as follows. Based on our previous LDA calculations, we found that the occupation of the Pd 5s band decreases near hydrogen atoms, whereas the filling of the Pd 4d band increases to a near-noble-metal configuration¹⁵. The latter effect decreases the contribution of 4d electrons to anisotropic bonding, reducing the shear modulus and increasing ductility. Similar behaviour is expected in other metals with a similar electronic configuration, such as Ni. (Our results can not be easily generalized to other systems with near half-filled d band, such as Fe. There, independent of hydrogen loading, the anisotropic d orbitals play a significant role in the mechanical response to applied tensile stress. The origin of hydrogen embrittlement in such systems is still an open question).

Hydrogen atoms have a very small mass and size, and an accordingly large diffusion coefficient. As hydrogen atoms are most stable in an expanded lattice, they accumulate rapidly near fluctuation- and stress-induced defects in the Pd matrix. Such defects are the mechanical weak points of the structure, as they can deform easily. This results in a locally enhanced ductility or plasticity.

In a system under tensile stress, the defects are often near strain singular points such as crack tips. Rapid accumulation of hydrogen at the crack tips results in a strongly inhomogeneous

hydrogen concentration, associated with local plasticity variations. Under tensile stress, fracture will occur at these hydrogen-saturated weak links, while the bulk of the matrix is not strongly affected. This mechanism leads to a fracture morphology which is indistinguishable from brittle fracture (because brittle fracture implies no crystal shape changes outside the crack region, whereas a typical ductile fracture is accompanied by large-scale shape deformations). Our findings are in agreement with the hydrogen-enhanced local plasticity mechanism of fracture¹⁰⁻¹², which had originally been proposed¹⁰ to explain fracture of hydrogen-loaded steel under tensile stress. Our results do not provide any evidence for the 'decohesion' or 'hydrogen-related phase-change' mechanisms of hydrogen embrittlement in PdH_x.

Although our numerical results have been obtained for idealized defect-free lattices, we expect the HELP mechanism also to apply in realistic systems. There, hydrogen accumulates preferentially in regions of extremely high local stresses, increasing ductility locally and allowing the system to relax by forming cracks. The presence of such cracks with ductile tips lowers the critical tensile stress substantially when compared to the defect-free system. In realistic crystals, deformation occurs by dislocation motion. The locally increased plasticity near hydrogen-loaded dislocations clearly lowers the friction stress for dislocation motion and hence the material's resistance to cleavage. It would be instructive to investigate experimentally the effect of extremely high stress loading rates on the critical tensile stress, and to test our corresponding predictions. □

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1. Beachem, C. D. *Hydrogen Damage* (American Society for Metals, Ohio, 1977).
2. Birnbaum, H. K., Grossbeck, M. & Gahr, S. in *Hydrogen in Metals* (eds Bernstein, M. & Thompson, A.) 303 (American Society for Metals, Ohio, 1973).
3. Birnbaum, H. K. in *Environmentally Sensitive Fracture of Engineering Materials* (ed. Foroulis, Z. A.) 326 (Metals Society, New York, 1979).
4. Steigerwald, E. A., Schaller, F. W. & Troiano, A. R. *Trans. metall. Soc. A.I.M.E.* **218**, 832 (1960).
5. Oriani, R. A. & Josephic, P. H. *Acta metall.* **22**, 1065-1074 (1974).
6. Oriani, R. A. & Josephic, P. H. *Acta metall.* **25**, 979-988 (1977).
7. Foiles, S. M., Baskes, M. I. & Daw, M. S. *Phys. Rev. B* **33**, 7983-7991 (1986).
8. Westlake, D. G. *Trans. Am. Soc. Metals* **62**, 1000 (1969).
9. Birnbaum, H. K. *J. Less Common Metals* **104**, 31-41 (1984).
10. Beachem, C. D. *Metall. Trans.* **3**, 437-451 (1972).
11. Tabata, T. & Birnbaum, H. K. *Scripta metall.* **18**, 231-236 (1984).
12. Lynch, S. P. *J. Mater. Sci.* **21**, 692-704 (1986).
13. Tománek, D., Louie, S. G. & Chan, C. T. *Phys. Rev. Lett.* **57**, 2594-2597 (1986).
14. Sun, Z. & Tománek, D. *Phys. Rev. Lett.* **63**, 59-62 (1989).

15. Tománek, D., Sun, Z. & Louie, S. G. *Phys. Rev. B* **43**, 4699-4713 (1991).
16. Allen, M. P. & Tildesley, D. J. *Computer Simulation of Liquids* (Oxford, New York, 1990).
17. Parrinello, M. & Rahman, A. *Phys. Rev. Lett.* **45**, 1196-1199 (1980).
18. Parrinello, M. & Rahman, A. *J. appl. Phys.* **52**, 7182-7190 (1981).
19. Ray, J. R. & Rahman, A. *J. chem. Phys.* **80**, 4423-4428 (1984).
20. Daw, M. S. & Baskes, M. I. *Phys. Rev. Lett.* **50**, 1285-1288 (1983).
21. Daw, M. S. *Phys. Rev. B* **39**, 7441-7452 (1989).
22. Pratt, L. R. & Eckert, J. *Phys. Rev. B* **39**, 13170-13174 (1989).
23. Landman, U., Luedtke, W. D., Burnham, N. A. & Colton, R. J. *Science* **248**, 454-461 (1990).
24. Zhong, W., Li, Y. S. & Tománek, D. *Phys. Rev. B* **44**, 13053-13062 (1991).
25. Zhong, W., Cai, Y. & Tománek, D. *Phys. Rev. B* **46**, 8099-8108 (1992).
26. Kittel, C. *Introduction to Solid State Physics* 6th Edn (Wiley, New York, 1986).
27. Peisl, H. *Hydrogen in Metals I*, 53-74 (Springer, Berlin, 1978).

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Determination of soil exchangeable-cation loss and weathering rates using Sr isotopes

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To assess the response of forests to a changing chemical environment, a means is needed for separating the total cation export from the watershed into a component derived from mineral weathering reactions and a component due to the removal of exchangeable (plant-available) cations in the soil¹⁻³. We show that this separation may be possible by using ⁸⁷Sr/⁸⁶Sr ratios as a tracer of cation sources in stream water. Our measurements from a high-elevation forest ecosystem in the Adirondack mountains, New York, indicate that mineral weathering reactions contribute about 70% and soil cation-exchange reactions about 30% of annual strontium exports. Based on these results and the ratios of major cations to strontium in the local glacial till, we estimate the release of Ca²⁺, Mg²⁺, K⁺ and Na⁺ owing to weathering. The present weathering rate seems adequate to replace annual losses of cations from the total soil exchangeable pool, suggesting that the watershed is not in immediate danger of acidification from atmospheric deposition. But as our strontium isotope data indicate that 50–60% of the strontium in the organic-soil-horizon exchangeable and vegetation cation pools has an atmospheric origin, reduction of atmospheric cation inputs⁴ coupled with continued strong-acid anion inputs⁵ may result in significant depletion of this cation reservoir.

The annual total cation export from soils in forested watersheds of the northeastern USA appears to have increased over the past 60 years owing to acid deposition^{2,6}. Measurements of atmospheric deposition to⁷, and nutrient export from^{8,9} a spruce-fir forest (1,000-m elevation) at Whiteface Mountain, New York, indicate an annual average nutrient cation (Ca²⁺, Mg²⁺, K⁺, Na⁺) denudation rate of 1.2 kmol(+) ha⁻¹ yr⁻¹ (kmol(+) indicates kilomoles of cationic charge). The soil exchangeable pool of nutrient cations at this site⁹ is ~40 kmol(+) ha⁻¹. If the entire cation loss from the ecosystem were derived from this soil exchange pool, it would be exhausted after ~30 yr. The contribution of mineral weathering reactions to (1) the cations lost in stream water and (2) the cations accumulated in vegetation and eventually transferred to the soil exchange pool must be determined in order to calculate the actual exchange pool depletion rate. These weathering fluxes of cations have not been measured previously. We used the isotopic composition of Sr, which takes part in chemical reactions in a similar way to Ca (ref. 10), to identify and quantify the contribution of different sources of Sr (and by inference Ca and other cations) present in vegetation and stream water.

Rocks develop values of the ⁸⁷Sr/⁸⁶Sr ratio which depend on the Rb/Sr ratio and the age of the rock, because ⁸⁷Sr is produced by the radioactive decay of ⁸⁷Rb (half-life, 49 Gyr) whereas ⁸⁶Sr is nonradiogenic. The ⁸⁷Sr/⁸⁶Sr ratio of the atmospheric aerosol is controlled by the ⁸⁷Sr/⁸⁶Sr ratio of the oceans¹¹ (0.70923 ± 0.00001) in coastal regions, whereas continental dust sources impart spatial and temporal variation to this value inland¹². Unlike H, C, N, O and S isotopes, Sr isotopes are not fractionated by biological or chemical processes¹⁰. The isotopic composition of Sr in various ecosystem cation pools represents mixing of Sr derived from atmospheric and mineral-weathering^{10,13-15}.

The study watershed has a soil parent material (glacial till ~1 m thick, deposited 12–14 kyr ago) that is dominated by the

local anorthosite bedrock which has a very low ⁸⁷Sr/⁸⁶Sr ratio (0.70478). The till also contains minor amounts of sandstone and metamorphic rocks derived from adjacent bedrock to the north and northeast¹⁶. Laboratory leachings of anorthosite bedrock and glacial till with 0.1 M HCl were conducted to simulate Sr release by weathering and produced solutions with average ⁸⁷Sr/⁸⁶Sr ratios of 0.705160 and 0.706585, respectively. The ⁸⁷Sr/⁸⁶Sr ratio of the atmospheric aerosol in this region, as indicated by our measurements of composite rainwater samples, is 0.70931. The Sr isotopic composition of vegetation, soil exchange pools and stream water in the spruce-fir forest at Whiteface Mountain illustrates mixing of atmospheric and mineral weathering sources (Table 1 and Fig. 1).

Isotope mixing equations may be solved for the fractional contribution of each end member to a two-component mixture if the isotopic compositions of each end member and the mixture are known. For this purpose we express the Sr isotopic composition of a sample as

$$\Delta^{87}\text{Sr} = \left[\frac{{}^{87}\text{Sr}}{{}^{87}\text{Sr} + {}^{86}\text{Sr}} \right]_{\text{sample}} - \frac{{}^{87}\text{Sr}}{{}^{87}\text{Sr} + {}^{86}\text{Sr}}_{\text{standard}} \times 10^4 \quad (1)$$

after Graustein^{10,13}, where the standard we have chosen is sea water. The fractional contribution x of end member A to a mixture (M) of A and B is given by

$$x = (\Delta^{87}\text{Sr}_M - \Delta^{87}\text{Sr}_B) / (\Delta^{87}\text{Sr}_A - \Delta^{87}\text{Sr}_B) \quad (2)$$

To use isotope mixing equations to partition Sr fluxes, the ecosystem must be divided into reservoirs of Sr (and other cations) which have distinct and readily measured ⁸⁷Sr/⁸⁶Sr ratios (Fig. 2).

Based on end-member ⁸⁷Sr/⁸⁶Sr ratios of rain water and of Sr released by 0.1 M HCl leaching of glacial till, ~53% of the Sr present in vegetation and 17–59% (O and BC horizons, respectively) of the Sr present in cation-exchange pools appears to have been derived from atmospheric sources (Fig. 1). This observation indicates that atmospheric deposition supplies a significant proportion of the nutrient cations in this ecosystem and that biological cycling processes efficiently retain atmospherically deposited cations in the plant-available pool. This result contrasts with the traditional view that mineral weathering reactions are the dominant source of available cations in soils of recently glaciated, temperate regions. The stream water Sr isotope data also indicate that significant chemical weathering of the anorthosite bedrock is occurring in the watershed (Fig. 1).

Using the Sr isotopic composition measurements from this study (Table 1), equations (1) and (2), and additional measurements of cation fluxes from a detailed nutrient cycling study⁷⁻⁹ (Table 2), we have estimated the cation-exchange and weathering release of Sr and major cations from soils in the spruce-fir forest at Whiteface Mountain (Fig. 2). Cation-exchange reactions contributed an average of 30%, and new mineral weathering reactions an average of 70%, of the Sr exported in stream water during five different measurement periods. New mineral weathering reactions apparently supply an average of 20% of the Sr incorporated into vegetation each year. The remaining 80% of annual uptake is supplied by recycled Sr (of both atmospheric and mineral weathering origin) residing in the soil-exchangeable pool. In Fig. 2b we show a preliminary calculation of Ca²⁺, Mg²⁺, K⁺ and Na⁺ fluxes in the ecosystem extrapolated from the cation/Sr²⁺ ratios of several reservoirs, some directly measured cation fluxes (Table 2) and the Sr fluxes from Fig. 2a. Additional research is required to characterize more fully the relative behaviour of Sr and major cations during many of the important biogeochemical processes occurring in this ecosystem in order to improve our calculations of the major cation fluxes.

On the basis of the inferred major cation weathering fluxes (Fig. 2b), we estimate that the pool of exchangeable cations in the organic soil horizon is roughly stable (<1% net annual

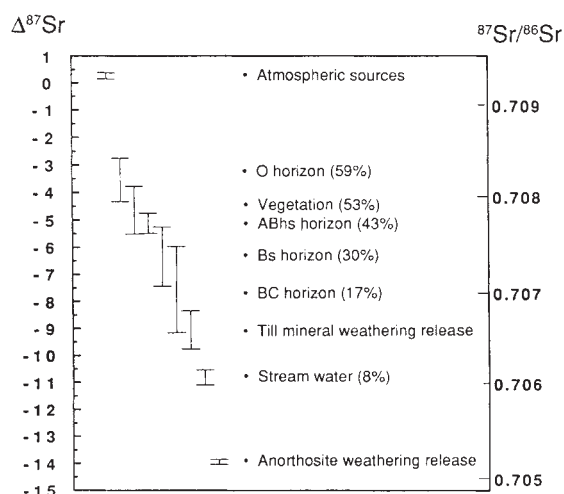


FIG. 1 Weighted average (solid circles) and one standard deviation (range bars) of the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio and $\Delta^{87}\text{Sr}$ of cation reservoirs in the spruce-fir forest at Whiteface Mountain. $\Delta^{87}\text{Sr}$ is calculated using equation (1) in the text. Analytical precision of individual measurements is smaller than the symbol size; the standard deviations reflect spatial variability (or temporal variability for atmospheric deposition and stream water) in the Sr isotopic composition of each reservoir. The vegetation value is a weighted average based on the relative contribution of each measured component to the total Ca content of the vegetation at the site⁹. Shown in parentheses is the percentage of Sr derived from atmospheric sources calculated with equation (2) in the text, assuming that the soil exchangeable-cation pools and vegetation cation pool are mixtures of cations derived from mineral weathering reactions in the glacial till ($\Delta^{87}\text{Sr} = -9.07$) and cations transported to the ecosystem in the atmospheric aerosol ($\Delta^{87}\text{Sr} = +0.27$). The $\Delta^{87}\text{Sr}$ of stream water is lower than that of Sr released from till and indicates a contribution from weathering of the anorthosite bedrock. We estimate from the calculations presented in Fig. 2 that ~8% of the Sr in stream water has an atmospheric source.

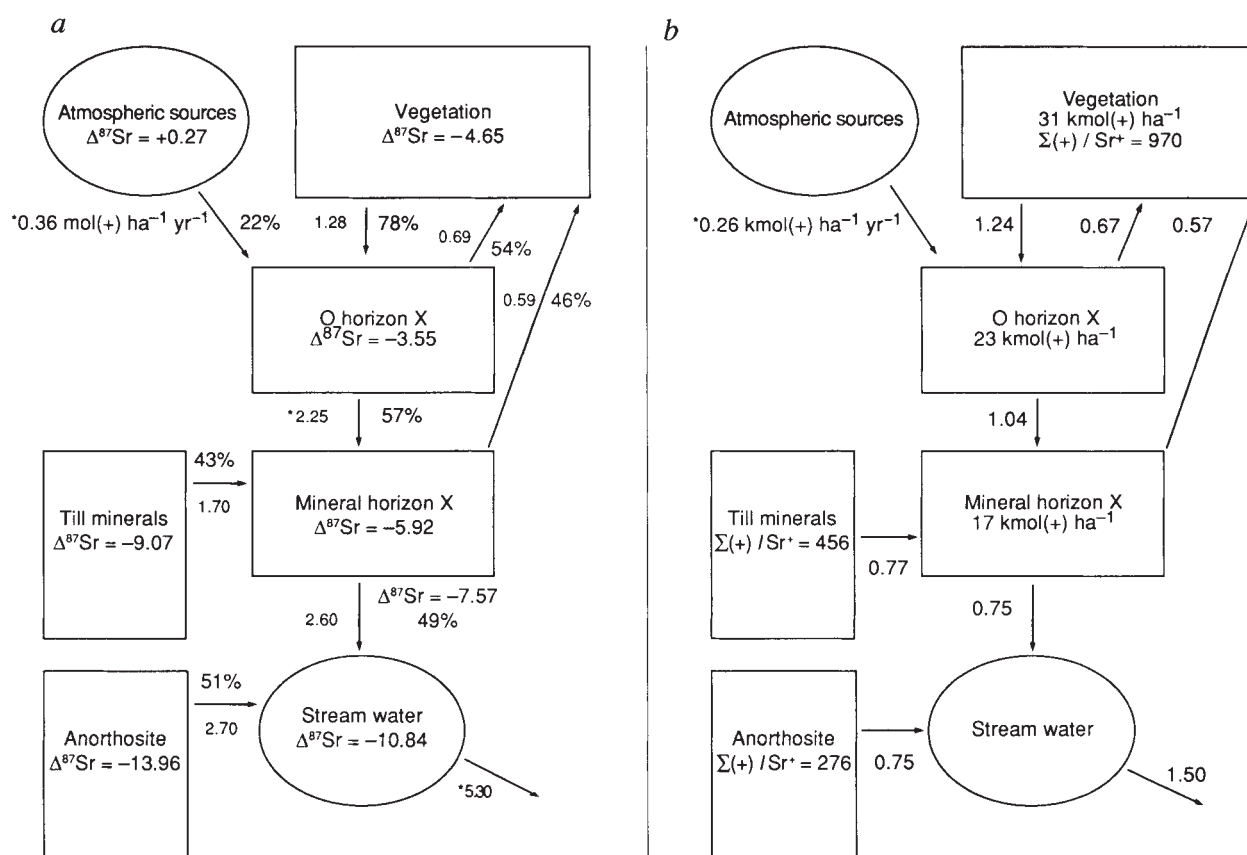


FIG. 2 First-order model of (a) Sr and (b) base cation fluxes in the spruce-fir ecosystem at Whiteface Mountain. *a*, The isotopic composition of each reservoir is given in $\Delta^{87}\text{Sr}$ units (see equation (1) in the text). The percentage contribution of each reservoir to a given Sr flux was determined by solving equation (2) in the text. Vegetation Sr was assumed to be a mixture of Sr extracted from the organic and mineral soil exchange pools. The organic horizon exchange pool receives Sr from atmospheric deposition and vegetative cycling (for example leaf litter). The exchangeable Sr in the mineral soil is assumed to be derived from till mineral weathering reactions and Sr transported by percolating soil water from (and in isotopic equilibrium with) the organic horizon. Stream water is assumed to obtain its Sr from both the BC soil horizon exchange pool and weathering of the anorthosite bedrock. Sr fluxes ($\text{mol}(+) \text{ ha}^{-1} \text{ yr}^{-1}$) between all reservoirs were calculated based on the relative contributions indicated by the isotopic mixing relationships

and a small number of Sr fluxes (indicated by asterisks) determined from the mass balance study⁷⁻⁹ (Table 2). *b*, Fluxes of Ca^{2+} , Mg^{2+} , Na^+ and K^+ ($\text{kmol}(+) \text{ ha}^{-1} \text{ yr}^{-1}$) calculated from the tracer-derived Sr fluxes and the ratio of $[\Sigma\text{Ca}^{2+} + \text{Mg}^{2+} + \text{Na}^+ + \text{K}^+]/\text{Sr}^{2+}$ for the flux (Table 2). The $\Sigma(+)/\text{Sr}^{2+}$ ratio for a flux was assumed to be the same as in the source reservoir except for vegetative uptake from the soil exchange pools, for which the ratio was assumed to be that of the vegetation reservoir. The $\Sigma(+)/\text{Sr}^{2+}$ ratio for cations released by weathering of till was assumed to be the ratio in material released since glaciation as determined from preliminary element depletion measurements. The total cation release from mineral weathering of till and bedrock is $\sim 1.5 \text{ kmol}(+) \text{ ha}^{-1} \text{ yr}^{-1}$ (weathered cations lost in stream water plus weathered cations stored on exchange sites or taken up by vegetation). The ecosystem appears to be accumulating cations at a rate of $\sim 0.3\text{--}0.5 \text{ kmol}(+) \text{ ha}^{-1} \text{ yr}^{-1}$.

TABLE 1 Isotopic compositions

Sample type	Sr	$^{87}\text{Sr}/^{86}\text{Sr}$	Sample type	Sr	$^{87}\text{Sr}/^{86}\text{Sr}$
Rain water	$\mu\text{g l}^{-1}$		Stream water	$\mu\text{g l}^{-1}$	
Jun–Sept. 1987*	n.a.	0.709345 ± 30	Sep/Oct 1987*	17.9	0.706382 ± 7
Jan–May 1988*	n.a.	0.709271 ± 48	Nov/Dec 1987*	42.1	0.706026 ± 6
31 May 1991	0.95	0.708380 ± 22	18 May 1991	14.7	0.706002 ± 24
18 July 1991	1.3	0.709045 ± 116	23 August 1991	26.0	0.705901 ± 21
31 July 1991	0.69	0.708483 ± 21	30 May 1992	NA	0.706037 ± 13
Soil exchangeable in 1M $\text{NH}_4(\text{OOCCH}_3)$	$\mu\text{g g}^{-1}$		Vegetation	$\mu\text{g g}^{-1}$	
O horizon (profile C4)	9.0	0.708424 ± 22	Spruce needles (tree C)	2.2	0.708867 ± 78
A horizon (profile C4)	0.54	0.707626 ± 20	Spruce needles (tree D)	NA	0.708742 ± 70
Bs horizon (profile C4)	0.34	0.707056 ± 27	Spruce wood (tree D)	NA	0.709101 ± 37
BC horizon (profile C4)	0.42	0.706564 ± 21	Spruce roots (tree C)	18.7	0.708211 ± 11
O horizon (profile C3)	6.7	0.707939 ± 18	Fir needles (tree A)	NA	0.709375 ± 47
A horizon (profile C3)	0.46	0.707814 ± 25	Fir needles (tree B)	7.7	0.707639 ± 54
Bs horizon (profile C3)	0.23	0.707718 ± 23	Fir wood (tree B)	8.3	0.707734 ± 259
BC horizon (profile C3)	0.29	0.707502 ± 21	Birch wood (tree E)	NA	0.707522 ± 19
Glacial till			Anorthosite		
0.1 M HCl leach			0.1 M HCl leach	10.9	0.705160 ± 16
ES-1	NA	0.706791 ± 30	Whole rock	706	0.704468 ± 28
ES-2	NA	0.706379 ± 23	Whole rock ¹⁷	587	0.7053 ± 10

Sr concentrations and isotope ratios of samples from the spruce-fir forest ecosystem on Whiteface Mountain. The isotope composition of Sr in each ecosystem compartment was determined by thermal-ionization mass spectrometry after clean-laboratory acid dissolution and quartz cation-exchange column chemical separation¹⁸. Reported uncertainties are 2σ in the last two or three decimal places resulting from measurement of 100–300 individual ratios. Sr isotopic composition was normalized to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$. Eight runs of NBS-987 during this study gave $^{87}\text{Sr}/^{86}\text{Sr} = 0.710274 \pm 0.000016$. Sr concentrations were determined by isotope dilution. Exchangeable Sr was extracted from air-dried soil using 1 M ammonium acetate prepared from double-quartz-distilled reagents. The total blank contributions were <1.2 ng Sr for 1 M ammonium acetate extracts, <42 pg Sr for other samples and are negligible. Asterisk indicates a composite of a series of samples taken over the indicated time period; NA, data not available.

TABLE 2 Major cation fluxes and ratio of major cations to Sr

	Present cation fluxes ($\text{mol}(+) \text{ha}^{-1} \text{yr}^{-1}$)					
	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Sr^{2+}	$\text{Sr}^{2+}/\text{Ca}^{2+}$
Atmospheric deposition	129	42	55	30	0.36	2.74×10^{-3}
O horizon soil water	632	272	117	20	2.25	2.16×10^{-3}
Stream water	1036	187	200	68	5.3	5.10×10^{-3}
	Present cation reservoir concentrations ($\text{mmol}(+) \text{kg}^{-1}$)					
	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Sr^{2+}	$\Sigma(+)/\text{Sr}^{2+}$
Vegetation	134	29.1	0.85	40.7	0.211	970
Anorthosite bedrock	3876	364	724	173	18.6	276
	Cations released since glaciation ($\text{mol}(+) \text{ha}^{-1} \text{yr}^{-1}$)					
	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Sr^{2+}	$\Sigma(+)/\text{Sr}^{2+}$
Till weathering	840	360	160	145	3.3	456

Atmospheric deposition fluxes represent (precipitation + cloudwater + dry deposition) and are averaged over the years 1986–1988 given in ref. 7, table 5. The atmospheric Sr flux is estimated from the Sr/Ca ratio in precipitation (Sr concentrations from Table 1 and Ca from ref. 7) and the total Ca flux. O horizon soil water fluxes represent the average flux (1986–1988) based on data from refs 8 and 9, table WF-2 and one additional year of unpublished data acquired using the methods given in refs 8 and 9. The O horizon soil water Sr flux was estimated from the Sr/Ca ratio in the exchangeable pool (Sr concentrations from Table 1 and Ca from unpublished data from the study described in refs 8 and 9) and the Ca flux. Stream water fluxes are unpublished data obtained with methods indicated in refs 8 and 9. Major cation concentrations in vegetation are unpublished data from the study described in refs 8 and 9. Strontium concentration in vegetation is from Table 1. In our first-order model of major cation fluxes (Fig. 2b) we assume that major cations and Sr are incorporated into vegetation from the organic and mineral horizon exchange pools and returned to the O horizon in the same proportions as found in the vegetation pool. Major cation and Sr concentrations in anorthosite bedrock were determined by X-ray fluorescence spectrometry (XRF). Weathering of anorthosite bedrock is assumed to release major cations and Sr in proportion to their abundances in the bulk rock, as anorthitic plagioclase comprises $>90\%$ of the rock. A preliminary element depletion study was conducted on soils developed from glacial till following the methods of April *et al.*⁶ The average element release rate (over the last 14 kyr) from till was calculated from XRF analyses of unweathered till and weathered residual (A and B soil horizons); soil mass estimates are unpublished data from the study described in refs 8 and 9. Present-day weathering of till minerals is assumed to release major cations and Sr in the same proportions as were calculated for the 14-kyr average weathering release.

depletion), whereas the mineral soil exchangeable-cation pool is growing at a rate of $\sim 0.5 \text{ kmol}(+) \text{ha}^{-1} \text{yr}^{-1}$ ($\Sigma \text{Ca}^{2+} + \text{Mg}^{2+} + \text{Na}^{+} + \text{K}^{+}$). The soils of this forested watershed seem to be reasonably well protected against the effects of anthropogenic acid deposition because of the abundance of readily weatherable plagioclase feldspar and hornblende. The present-day weathering rate of glacial till calculated from the Sr isotope data appears to be lower than the long-term average weathering rate (1.1 – $1.7 \text{ kmol}(+) \text{ha}^{-1} \text{yr}^{-1}$) since glaciation ~ 14 kyr ago, calculated for the same soil/till profiles on the basis of element depletion

measurements following April *et al.*⁶.

A decrease in the input of cations to ecosystems from atmospheric sources has been observed since 1965 (refs 4, 19). As our results show that (for strontium at least) these sources have contributed a significant proportion of the total annual input to the plant-available reservoir, further decreases in atmospheric inputs of cations coupled with a stabilized or increased flux of strong acid anions⁵ through the organic soil horizon may ultimately deplete the reservoir of cations most readily available to vegetation. □

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- Wright, R. F. in *Physical and Chemical Weathering in Geochemical Cycles* (eds Lerman, A. & Meybeck, M.) 181–196 (Kluwer, Dordrecht, 1988).
- Federer, C. A. et al. *Envir. Mgmt.* **13**, 593–601 (1988).
- Johnson, D. W. et al. *Proc. R. Soc. Edinb.* **97B**, 81–116 (1991).
- Likens, G. E. et al. *Atmos. Envir.* **18**, 2641–2647 (1984).
- James, B. R. & Riha, S. J. *J. envir. Qual.* **15**, 229–234 (1986).
- April, R., Newton, R. & Coles, L. *Geol. Soc. Am. Bull.* **97**, 1232–1238 (1986).
- Miller, E. K., Panek, J. A., Friedland, A. J., Kadlecik, J. & Mohnen, V. A. *Tellus* (in the press).
- Friedland, A. J., Miller, E. K., Battles, J. J. & Thorne, J. F. *Biogeochemistry* **14**, 31–55 (1991).
- Friedland, A. J. & Miller, E. K. in *Atmospheric Deposition and Forest Nutrient Cycling* (eds Johnson, D. & Lindberg, S.) (Springer, New York, 1992).
- Graustein, W. C. *Stable Isotopes in Ecological Research* 491–512 (Springer, New York, 1989).
- DePaolo, D. J. & Ingram, B. L. *Science* **227**, 938–941 (1985).
- Andersen, P., Lofvendahl, R. & Åberg, G. *Atmos. Envir.* **24A**, 2601–2608 (1990).
- Graustein, W. C. & Armstrong, R. L. *Science* **219**, 289–292 (1983).
- Gosz, J. R., Brooks, D. G. & Moore, D. I. *Bioscience* **33**, 23–30 (1983).
- Åberg, G., Jacks, G. & Hamilton, P. J. *J. Hydrol.* **109**, 65–78 (1989).
- Craft, J. L. Ph.D. thesis. Univ. of Western Ontario (1976).
- Heath, S. A. & Fairbairn, H. W. in *Origin of Anorthositic and Related Rocks* (ed. Isachsen, Y.W.) (New York State Museum and Science Service, Albany, 1969).
- Papanastassiou, D. A., DePaolo, D. J. & Wasserburg, G. J. *Proc. 8th Lunar Sci. Conf.* 1639–1672 (1977).
- Driscoll, C. T., Likens, G. E., Hedin, L. O., Eaton, J. S. & Bormann, F. H. *Envir. Sci. Technol.* **23**, 137–143 (1989).

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Evidence from hafnium isotopes for ancient sub-oceanic mantle beneath the Rio Grande rift

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UNDERSTANDING the nature of the mantle underlying continents is important for balancing the inventory of the Earth's main chemical reservoirs^{1,2} and for constraining aspects of mantle convection, such as the extent to which continental lithosphere is coupled to the asthenosphere beneath^{3–5}. Strontium, neodymium and lead isotope studies of basaltic lavas from regions of continental extension, such as the southwestern United States, have identified at least two components of the sub-continental mantle⁶: one composed of ancient lithosphere, enriched by the addition of metasomatic fluids or crustal material, and an asthenospheric component with Sr, Nd and Pb isotope compositions matching those of ocean-island basalts. The lutetium–hafnium isotope system^{7–9} provides additional information not obtainable from the other systems; in particular, by betraying the presence of garnet during melting^{10,11} (hence constraining the depth of previous melting events of the source mantle). Here we report that basalts derived from upwelling asthenosphere in the region of the Rio Grande rift, southwestern United States, have Nd and Hf isotope ratios that lie significantly off the ocean-island Nd–Hf array¹⁰. We interpret the low ¹⁷⁶Hf/¹⁷⁷Hf ratios in these basalts as reflecting derivation from ancient asthenospheric mantle that melted at shallow levels beneath the oceans. Hafnium isotopes in continental basalts may thus provide evidence for large-scale overriding by continents of sub-oceanic mantle.

It is well established that ¹⁴³Nd/¹⁴⁴Nd and ¹⁷⁶Hf/¹⁷⁷Hf ratios of modern ocean-island basalts (OIB) are well correlated, reflecting correlated variations in Sm/Nd and Lu–Hf parent-daughter ratios that must have been established several billion years ago to produce systematic variations in present-day radiogenic isotope ratios^{7–9}. The Nd–Hf OIB array (Fig. 1) has been interpreted to reflect mixing between a depleted mantle component that may have been the residue of melt extraction, and an enriched component that was produced by metasomatism or

contamination with crustal material¹⁰. In contrast, Nd and Hf isotope variations in the mantle sources for mid-ocean-ridge basalts (MORB) are not correlated^{7,8,10}, indicating markedly different fractionation of Sm/Nd and Lu/Hf ratios during ancient depletion events in the mantle. The Lu/Hf partition coefficient ratio ($D_{Lu/Hf}$) is dramatically higher for garnet (≥ 24) than, for example, for clinopyroxene ($D_{Lu/Hf} \approx 1$); this contrast indicates the strong role garnet may play in changing the Lu/Hf ratio of the mantle during melting events^{10,11}. The presence or absence of garnet during ancient depletion events would therefore produce distinctly different present-day Hf isotope ratios. Only a few Hf isotope determinations of continental basalts have been made¹⁰, and these data are important for comparing the depletion history and origin depths of the sub-continental mantle with that of the sub-oceanic mantle.

We report Hf isotope compositions for 25 lavas from the Rio Grande rift in the southwestern United States; these lavas represent the entire range of Sr, Nd and Pb isotope compositions that has been identified for the mantle in the rift and southern Basin and Range ($\epsilon_{Nd} = -4$ to $+9$); $\epsilon_{Nd} = [({}^{143}\text{Nd}/{}^{144}\text{Nd})_{\text{sample}}/({}^{143}\text{Nd}/{}^{144}\text{Nd})_{\text{CHUR}} - 1] \times 10^4$, where CHUR refers to chondrite uniform reservoir and are therefore thought to sample a range of lithospheric and asthenospheric mantle compositions^{12–14}. Early extension lavas (from ~26 Myr ago; San Luis Hills) may represent largely lithospheric mantle compositions¹⁴, whereas younger lavas, particularly in the southern rift region (examples are Geronimo and Mogollon–Datil), are generally thought to be dominated by an asthenospheric mantle component^{12,13}. Most of the lavas studied here

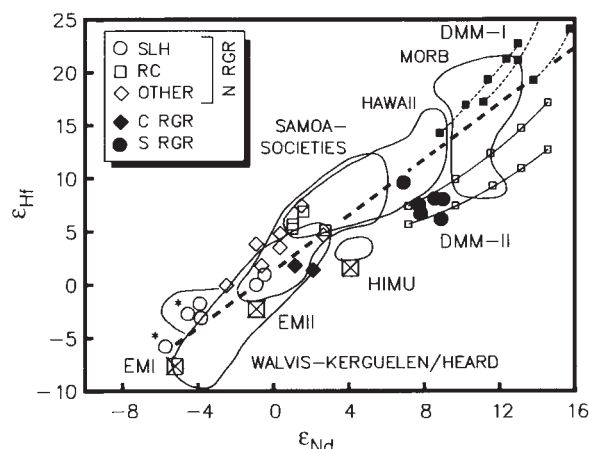


FIG. 1 Nd and Hf isotope ratios for late Cenozoic lavas from the Rio Grande rift and southern Basin and Range, divided as southern rift region (S RGR), central rift (C RGR), and northern rift (N RGR); symbols for northern samples further divided into San Luis Hills (SLH) and Rayton-Clayton (RC) suites. Two crustally contaminated samples from SLH marked with asterisk next to symbol. Nd and Hf isotope data for continental lavas at Leucite Hills¹⁰, which crop out in Southern Wyoming, have very negative ϵ_{Nd} (< -10) and positive $\Delta\epsilon_{Hf}$ (up to $+6$), which are isotopic compositions that may reflect ancient metasomatism⁴². Fields for oceanic lavas shown; all oceanic data fall within the fields^{7–10,16,18,24,25}. EM I, EM II and HIMU components from ref. 10; DMM I and DMM II components from this paper. The HIMU component is located at the proposed extreme endmember position¹⁰. Long dashed line indicates ocean-island basalt (OIB) regression line, which was calculated using all published data, except for Hawaii and HIMU components; half of all OIB Hf data are from Hawaii, and the regression equally weighted the Koolau, Kea and Posterosional components of ref. 24 for a total of 15 points, which balances the 15 low- ϵ_{Nd} points of Kerguelen and Kerguelen–Heard Plateau. If all the Hawaiian data are used, the slope of the regression changes from 1.36 to 1.35. Depleted mantle curves are shown in upper right, illustrating depletion of garnet peridotite (upper set, solid squares) and depletion of spinel peridotite (lower set, open squares); see Fig. 2 for details. On the basis of duplication of over 1/3 of the Rio Grande rift analyses, Hf isotope ratios are precise to $\pm 0.8 \epsilon_{Hf}$ units; this is slightly larger than the size of the symbols. All ocean and continental lava Hf data are normalized to JMC-475 $\epsilon_{Hf} = -23.47$ (see Table 1).

TABLE 1 Isotope and chemical data for Cenozoic lavas from the Rio Grande rift

Sample	Rock	SiO ₂	MgO	TiO ₂	Ni	Cr	Sr	Zr	Lu	Hf	¹⁷⁶ Hf/ ¹⁷⁷ Hf	ε _{Hf}	Δε _{Hf}	Sm	Nd	¹⁴³ Nd/ ¹⁴⁴ Nd	ε _{Nd}	Ref.
SOUTHERN RIO GRANDE RIFT REGION																		
<i>Geronimo volcanic field</i>																		
PKGRF-26	BAS	45.9	6.98	2.02	85		826	248			0.283000 ± 59	6.1	-7.5			0.513072	8.8	12, 32*
PKG-34	AOB	48.5	8.05	1.87	154	236	502	164			0.283018 ± 42	6.8	-5.6				7.9	12, 32*
PKGD-12	BAS	46.6	8.81	2.10	152	286	553	239	0.37	4.9	0.283032 ± 67	7.2	-4.9	5.97	25	0.513014	7.7	12, 32*
PKGD-55	BAS	45.9	10.03	2.21	200	342	685	219	0.41	4.6	0.283021 ± 93	6.9	-6.4	6.27	36	0.513059	8.6	12, 32*
											0.283051 ± 61	7.9	-5.4					
<i>Eastern Mogollon-Datil volcanic field</i>																		
GR-30C	TRB	48.6	6.03	2.04	123	111	810	226	0.32	4.7	0.283097 ± 54	9.5	-1.5	6.67	31		6.9	†
											0.283051 ± 122	7.9	-3.1					
T-4-81	AOB	46.9	8.43	2.65	183	224	697	217	0.34	4.5	0.283015 ± 33	6.6	-7.3	6.34	28		9.0	†
											0.283054 ± 18	8.0	-5.8					
CENTRAL RIO GRANDE RIFT																		
<i>Eastern Mount Taylor volcanic field</i>																		
63	OT	50.0	8.73	1.29	233	240	103				0.282875 ± 42	1.7	-1.4	3.24	12.5		1.1	13
<i>Espanola Basin:</i>																		
89SB-219	AOB	43.1	13.09	2.48	317	569	808	235	0.26	7.5	0.282867 ± 78	1.4	-3.0	12.29			2.1	‡
											0.282862 ± 34	1.2	-3.2					
NORTHERN RIO GRANDE RIFT																		
<i>Taos Plateau volcanic field</i>																		
RG-335	OT	50.1	8.21	1.31	151	227	371	89	0.28	2.3	0.282960 ± 17	4.7	2.7	3.11	11.6		0.3	33§
RG-154	TRB	51.5	6.03	1.42	93	140	1,025	215	0.31	4.6	0.282924 ± 20	3.4	1.4	6.63	36.5		0.3	33§
<i>Los Mogotes volcano</i>																		
BB-2	TRB	51.4	6.67	1.61	115	153	981	180	0.31	4.4	0.283038 ± 49	7.5	3.9	5.77	26		1.4	
BB-3	TRB	51.6	6.47	1.61	100	132	996	176	0.31	4.3	0.282960 ± 83	4.7	-0.5	5.82	27		2.6	
<i>Rayton-Clayton volcanic field</i>																		
855-116	NE	41.3	8.32	1.99	119	119	1,988	272	0.46	5.6	0.282982 ± 41	5.5	2.5	16.90	107		1.0	34, 35¶
855-271	BAS	44.8	9.85	1.87	215	276	1,401	183	0.39	3.9	0.283011 ± 71	6.5	3.5	9.20	55		1.0	33, 35¶
											0.282986 ± 26	5.6	2.6					
855-380	BAS	44.5	9.67	1.93	207		1,453	215			0.283011 ± 28	6.5	2.7			0.512700	1.6	33, 35¶
											0.283022 ± 27	6.9	3.1					
855-1861	NE	40.7	9.27	2.10	159		2,195	236			0.282972 ± 85	5.1	-0.3			0.512761	2.8	33, 35¶
<i>Amalia</i>																		
Q83J-107	BAS	45.6	8.65	2.69	152	213	1,820	219			0.282825 ± 28	-0.1	1.7	11.37	69.66		-2.5	36, 37
Q83J-108	AOB	49.9	7.94	1.71	128	210	575	129			0.282877 ± 28	1.8	1.0	5.37	24.01		-0.6	36, 37
											0.282912 ± 45	3.0	2.2					
Q83J-115	AOB	49.0	8.66	1.73	137	220	530	119			0.282936 ± 48	3.9	3.4	5.06	22.57		-0.9	36, 37
<i>San Luis Hills</i>																		
T84-152	TRB	49.4	8.08	1.58		289	683	151	0.25	4.4	0.282776 ± 30	-1.8	1.9	7.48	54.1		-3.9	14, 38
T84-98	TRB	49.3	8.11	1.55		338	710	149	0.28	4.1	0.282740 ± 37	-3.1	0.5	6.98	47		-3.8	14, 38
T84-140	TRB	50.3	3.73	2.13		22	1,135	220	0.35	5.3	0.282826 ± 33	0.0	-0.4	8.13	55		-0.9	14, 38
											0.282835 ± 35	0.3	-0.1					
T84-89	OT	50.0	6.87	1.59		173	395	116	0.27	2.9	0.282870 ± 71	1.5	0.4	4.35	23		-0.4	14, 38
											0.282853 ± 70	0.9	-0.2					
T84-150	AND	56.9	4.37	1.30		129	886	173	0.20	4.4	0.282749 ± 24	-2.8	1.9	6.53	47		-4.6	14, 38
T84-125	AND	57.7	4.11	1.21		121	891	208	0.22	5.2	0.282659 ± 25	-5.9	0.3	8.10	48		-5.8	14, 38

Forty-seven analyses of JMC-475 at the University of Wisconsin, Madison between 1990 and 1992 produced an average ¹⁷⁶Hf/¹⁷⁷Hf ratio of 0.282163 ± 10 (2 s.e.), which is equivalent to ¹⁷⁶Hf/¹⁷⁷Hf for CHUR of 0.282827. This is equivalent to present-day chondrite (CHUR) ¹⁷⁶Hf/¹⁷⁷Hf = 0.282862 and JMC-475 ¹⁷⁶Hf/¹⁷⁷Hf = 0.282198 (refs 39, 40), where JMC-475 has ε_{Hf} = -23.47. Nine samples were run in duplicate (separate chemical separation and mass spectrometry), and average reproducibility is ±0.8 ε_{Hf} units. Internal run statistics (2 s.e.) seem pessimistic, being on average twice as large as the average error of duplicate analyses. Details of analytical methods for Hf are given elsewhere⁴¹. ¹⁴³Nd/¹⁴⁴Nd ratios listed were measured at the University of Wisconsin, Madison; analytical details for Nd given elsewhere^{14,41}. AND, andesite; OT, olivine tholeiite; TRB, trachybasalt; AOB, alkali-olivine basalt; BAS, basalt; NE, nephelinite. SiO₂, MgO, TiO₂ in wt %; Ni, Cr, Sr, Zr, Lu, Hf, Sm, Nd in p.p.m. ε_{Nd} for 855-116 is estimated from other samples of similar composition.

* Also from P. Kempton, unpublished data.

† J. Ratte and K. Futa, unpublished data.

‡ S. Gibson, unpublished data.

§ S. Moorbath and M. Dungan, unpublished data.

|| J. Davidson and M. Dungan, unpublished data.

¶ J. Stormer, unpublished data.

have been shown in previous studies to be free of significant crustal contamination; high MgO, Ni and Cr contents, and low Sr and Zr contents, within related suites identify the least fractionated and least crustally contaminated samples (Table 1). Four of the samples (GR-30C, RG-154, T84-140 and T84-89) may contain up to a few per cent crust as shown by Pb isotope studies, although the amount of crust is not detectable in Nd or Hf isotope compositions. Two andesites contain 10–20% crust (T84-150, T84-125; ref. 14), and the decrease from mantle values in ε_{Hf} units (4 and 7, respectively) is comparable to the decrease in ε_{Nd} units.

In this work and in other studies under way on additional continental lavas¹⁵, Nd-Hf isotope variations in some cases differ from those of OIB, and we define an oceanic Nd-Hf correlation line as a reference (Fig. 1). We have regressed all published OIB Nd and Hf isotope data (Fig. 1) and here define a parameter Δε_{Hf}, which is the difference between the measured

ε_{Hf} value and that predicted by the OIB Nd-Hf correlation

$$\epsilon_{\text{HF}}(\text{OIB}) = 1.36\epsilon_{\text{Nd}} + 1.63$$

The correlation coefficient (R^2) is 0.87, and the errors in the slope and intercept (1 s.d.) are ±0.07 and ±2.47, respectively. Over 90% of the 76 published OIB Hf analyses (excluding HIMU) lie within ±3.1 Δε_{Hf} units (Fig. 2). The EM I and EM II components¹⁰ lie at a slightly negative Δε_{Hf} value, whereas the average for island arcs lies at a slightly positive Δε_{Hf} value (Fig. 2b). We have omitted the HIMU component¹⁰ from the regression because it lies off the main ocean Nd-Sr array¹⁶. Moreover, HIMU seems to be the only OIB component that lies significantly off the main Nd-Hf OIB array. Previously it has been noted¹⁷ that the slope of the Nd-Hf OIB array could be produced by mixing depleted mantle with subducted sediment that was a mixture of turbidite sandstone (low Lu/Hf) and pelagic sediment (high Lu/Hf). The significantly negative

$\Delta\epsilon_{\text{Hf}}$ value of HIMU is consistent with a negligible pelagic sediment component, as has been suggested on the basis of Pb isotope variations¹⁸. The spread in $\Delta\epsilon_{\text{Hf}}$ units for MORB, which is very large in relation to the change in ϵ_{Nd} values (Fig. 2), prompts us to define two depleted mantle components, which we here refer to as DMM I (high $\Delta\epsilon_{\text{Hf}}$) and DMM II (low $\Delta\epsilon_{\text{Hf}}$).

The contrast in negative and positive $\Delta\epsilon_{\text{Hf}}$ values for depleted (high ϵ_{Nd}) mantle is well explained by ancient melting (deple-

tion) of spinel and garnet peridotite, respectively (Fig. 2). Ancient melting without garnet is required to explain the very low present-day $\Delta\epsilon_{\text{Hf}}$ values of the DMM II component, which restricts these melting events to the uppermost 80 km of the mantle¹⁹. In contrast, the positive present-day $\Delta\epsilon_{\text{Hf}}$ values of the DMM I component are well explained by ancient depletion of garnet peridotite, and the range in isotopic compositions can be produced through small variations in the relative proportions of garnet and clinopyroxene (Fig. 2). The OIB Nd-Hf array, which appears to extend into the DMM I component, is best explained by ancient depletion events that occurred in the garnet peridotite stability field. Although small variations in distribution coefficients can have large effects in calculated elemental abundances, and lesser effects on Lu/Hf and Sm/Nd ratio abundances, the differences in Sm/Nd and Lu/Hf ratios calculated for depletion of spinel and garnet peridotites are relatively constant; as calculated over a range of D values and source and melting modes, depleted garnet peridotites always have higher $\Delta\epsilon_{\text{Hf}}$ values than those of depleted spinel peridotites, and we consider the petrological interpretation of the high and low $\Delta\epsilon_{\text{Hf}}$ components to be robust. Because depletion of garnet or spinel peridotite produces virtually the same Sm/Nd ratios under a given set of conditions, the Nd isotope ratios cannot tell us the mineralogy that was involved during ancient mantle depletion.

Basaltic lavas from the southern Rio Grande rift (Mogollon-Datil) and adjacent Basin and Range (Geronimo) have Nd and Hf isotope compositions similar to those of the DMM II component, although they are displaced to slightly lower ϵ_{Nd} and higher $\Delta\epsilon_{\text{Hf}}$ values (Fig. 2), possibly reflecting less depletion or the presence of an enriched component. The Nd and Hf isotope compositions of the southern lavas can be well explained if they were largely derived from asthenospheric mantle that was depleted in the spinel peridotite stability field 1–2 Gyr ago (Fig. 2). Recent geophysical studies have shown that the asthenosphere beneath the southern rift region is very shallow^{20,21}, and previous Nd isotope studies have also suggested that the southern lavas were derived from upwelling asthenosphere¹³. It seems unlikely that depletion of lithospheric mantle at shallow depths beneath the continents could explain the negative $\Delta\epsilon_{\text{Hf}}$ values of the RGR lavas, given the evidence that recent mantle upwelling has probably replaced most of the lithospheric mantle^{13,20,21}. Moreover, to the extent that metasomatism and contamination occur in much of the uppermost subcontinental mantle during early formation of the lithosphere¹⁴, it is unlikely that such parts of the lithospheric mantle would retain a depleted Nd and Hf isotope composition. We therefore propose that the depletion took place in sub-oceanic mantle that was subsequently over-ridden by the North American continent.

Northern and central rift basaltic lavas have Nd and Hf isotope compositions that lie within the OIB field, although the $\Delta\epsilon_{\text{Hf}}$ values of the northern samples are displaced to positive values relative to the EM I and EM II components (Fig. 2). The northern rift lavas are more likely to record a lithospheric mantle composition, as the lithosphere is thicker than for the southern region^{22,23}, whereas central rift samples may represent a mixture of asthenosphere and lithosphere, as suggested by previous Nd isotope studies¹³. Because the northern rift lavas have ϵ_{Nd} and $\Delta\epsilon_{\text{Hf}}$ values near zero, the presumed starting point for depletion of both spinel or garnet peridotite, we cannot deduce the depth of ancient depletion events for these sources. Previous models have suggested that the low ϵ_{Nd} values of the lithospheric mantle in the northern rift, as compared with those of depleted mantle, were generated by metasomatic or contamination processes in a Proterozoic arc setting¹⁴; if this model is valid, decreases in $^{176}\text{Hf}/^{177}\text{Hf}$ ratios during ancient metasomatism or contamination must have correlated with decreases in $^{143}\text{Nd}/^{144}\text{Nd}$ ratios.

Occurrence of the DMM II (negative $\Delta\epsilon_{\text{Hf}}$) component beneath the continents places constraints on the extent of asthenosphere–lithosphere coupling, which has been extensively

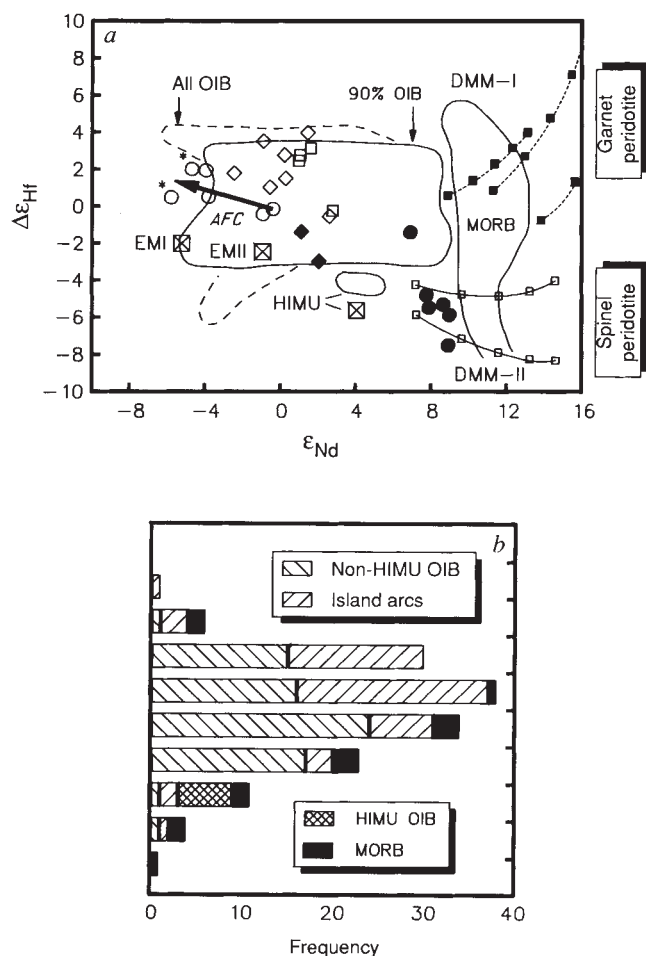


FIG. 2 *a*, $\Delta\epsilon_{\text{Hf}} - \epsilon_{\text{Nd}}$ variations for Rio Grande rift lavas, OIB and MORB. AFC notes crustal contamination trend based on two analyses of San Luis Hills andesites (marked with asterisk). Mantle depletion with ancient melt extraction from garnet peridotite produces high present-day $\Delta\epsilon_{\text{Hf}}$ values (short-dashed curves, solid squares), whereas ancient depletion of spinel peridotite produces low present-day $\Delta\epsilon_{\text{Hf}}$ values (solid curves, open squares). All depletion curves were calculated assuming melt extraction at 2 Gyr ago from chondritic mantle and non-modal equilibrium melting; modes and melting fractions are from ref. 26. D values from: garnet and clinopyroxene (favoured set of ref. 10), olivine²⁷, spinel²⁶ (D_{Hf} set equal to D_{Sm}), orthopyroxene²⁸. Garnet peridotite depletion curves calculated for modal garnet of 5% (uppermost curve), 8% and 11% (lowermost curve). Boxes shown for 1% melt increments, starting at 4% (left end of curves). Modal garnet + clinopyroxene kept equal to 23% (ref. 26). For spinel peridotite depletion curves, boxes shown for 2% melt increments, starting at 4% (left end of curve). One of the largest effects of changing D values lie in D_{Hf} for orthopyroxene (opx) in spinel peridotite due to the large melting fraction of opx; for upper spinel curve, opx $D_{\text{Hf}} = 0.04$ (ref. 28). However, recent ion probe studies of opx have shown very high abundances of Zr (and, by inference, Hf)²⁹, and the lower spinel curves was arbitrarily calculated assuming $D_{\text{Hf}} = 0.10$. We emphasize that regardless of the D_{Hf} value chosen for opx, depletion of spinel peridotite always produces lower $\Delta\epsilon_{\text{Hf}}$ values than depletion of garnet peridotite. *b*, $\Delta\epsilon_{\text{Hf}}$ histogram (same scale as in *a*) for OIB, MORB and island arcs. Data sources cited in Fig. 1; additional data from refs 30, 31.

debated³⁻⁵. Although recent discussions on mantle convection and the driving forces for plate motions have favoured plate velocities that are similar to mantle convective velocities⁵, further documentation of the DMM II component beneath several continents of distinct tectonic and accretionary history would indicate that asthenosphere-lithosphere slip represents a significant proportion of absolute plate velocities, particularly beneath relatively slowly moving continents⁴. □

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- Hawkesworth, C. J., Kempton, P. D., Rogers, N. W., Ellam, R. M. & van Calsteren, P. W. *Earth planet. Sci. Lett.* **96**, 256-268 (1990).
- McDonough, W. F. *Earth planet. Sci. Lett.* **101**, 1-18 (1990).
- Richardson, R. M., Solomon, S. C. & Sleep, N. H. *Rev. Geophys. Space Phys.* **17**, 981-1019 (1979).
- Gordon, R. G. & Jurdy, D. M. *J. geophys. Res.* **91**, 12389-12406 (1986).
- Davies, G. F. & Richards, M. A. *J. Geol.* **100**, 151-206 (1992).
- Kempton, P. D., Fitton, J. G., Hawkesworth, C. J. & Ormerod, D. S. *J. geophys. Res.* **92**, 13713-13735 (1991).
- Patchett, P. J. & Tatsumoto, M. *Geophys. Res. Lett.* **7**, 1077-1080 (1980).
- Patchett, P. J. *Lithos* **15**, 47-51 (1983).
- Patchett, P. J. *Geochim. cosmochim. Acta* **47**, 81-91 (1983).
- Salters, V. J. M. & Hart, S. R. *Earth planet. Sci. Lett.* **104**, 364-380 (1991).
- Salters, V. J. M. & Hart, S. R. *Nature* **342**, 420-422 (1989).
- Menzies, M. A., Leeman, W. P. & Hawkesworth, C. J. *Nature* **303**, 205-209 (1983).
- Perry, F. V., Baldrige, W. S. & DePaolo, D. J. *J. geophys. Res.* **92**, 9193-9213 (1987).
- Johnson, C. M. & Thompson, R. A. *J. geophys. Res.* **96**, 13593-13608 (1991).
- Barovich, K. M. & Johnson, C. M. *Science* (submitted).
- Hart, S. R., Gerlach, D. C. & White, W. M. *Geochim. cosmochim. Acta* **50**, 1551-1557 (1986).
- Patchett, P. J., White, W. M., Feldmann, H., Kielinczuk, S. & Hofmann, A. W. *Earth planet. Sci. Lett.* **69**, 365-378 (1984).
- Chauvel, C., Hofmann, W. A. & Vidal, P. *Earth planet. Sci. Lett.* **110**, 99-119 (1992).
- Takahashi, E. & Kushiro, I. *Am. Mineral.* **68**, 859-879 (1983).
- Olsen, K. H., Baldrige, W. S. & Callendar, J. F. *Tectonophysics* **143**, 119-139 (1987).
- Sinno, Y. A., Dattet, P. H., Keller, G. R., Morgan, P. & Harder, S. H. *J. geophys. Res.* **91**, 6143-6156 (1986).
- Davies, P. M., Parker, E. C., Evans, J. R., Iyer, H. M. & Olsen, K. H. *New Mexico geol. Soc. Guidebook* **35**, 29-38 (1984).
- Iyer, H. M. & Hitchcock, T. *Geol. Soc. Am. Mem.* **144**, 179-209 (1989).
- Stille, P., Unruh, D. M. & Tatsumoto, M. *Geochim. cosmochim. Acta* **50**, 2303-2319 (1986).
- Stille, P., Unruh, D. M. & Tatsumoto, M. *Nature* **304**, 25-29 (1983).
- Ottone, G., Ernst, W. G. & Joron, J. L. *J. Petrol.* **25**, 343-372 (1984).
- Fujimaki, H., Tatsumoto, M. & Aoki, K. *J. geophys. Res.* **89**, 662-672 (1984).
- Irving, A. J. & Frey, F. A. *Geochim. cosmochim. Acta* **48**, 1201-1221 (1984).
- Rampone, E., Bottazzi, P. & Ottoloni, L. *Nature* **354**, 518-520 (1991).
- White, W. M. & Patchett, P. J. *Earth planet. Sci. Lett.* **67**, 167-185 (1984).
- Woodhead, J. D. *Chem. Geol.* **76**, 1-24 (1989).
- Kempton, P. D., Dungan, M. A. & Blanchard, D. P. *Geol. Soc. Am. Spec. Pap.* **215**, 347-370 (1987).
- Dungan, M. A. et al. *J. geophys. Res.* **91**, 5999-6208 (1986).
- Phelps, D. W., Gust, D. A. & Wooden, J. L. *Contrib. Miner. Petrol.* **84**, 182-190 (1983).
- Stormer, J. C. Jr. *Geol. Soc. Am. Bull.* **83**, 2443-2448 (1972).
- Johnson, C. M. & Lipman, P. W. *Contrib. Miner. Petrol.* **100**, 107-128 (1988).
- Johnson, C. M., Lipman, P. W. & Czamanske, G. K. *Contrib. Miner. Petrol.* **104**, 99-124 (1990).
- Thompson, R. A., Johnson, C. M. & Mehnert, H. H. *J. geophys. Res.* **96**, 13577-13592 (1991).
- Patchett, P. J. & Tatsumoto, M. *Nature* **288**, 571-574 (1980).
- Tatsumoto, M., Unruh, D. M. & Patchett, P. J. *Proc. 6th Symp. Antarctic Meteorites* 237-249 (Nat. Inst. Polar Res., Tokyo, 1981).
- Beard, B. L., thesis, Univ. Wisconsin-Madison (1992).
- Beard, B. L. & Johnson, C. M. *Earth planet. Sci. Lett.* (submitted).

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Evolutionary significance of introgressive hybridization in cyprinid fishes

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BOTANISTS have long recognized introgressive hybridization as important in the evolution of plants^{1,2}. Hybridization among animals is also common³, yet few zoologists have considered it evolutionarily important⁴⁻⁶. We report here that in the genus *Gila*, a morphologically diverse group of minnows from western North America⁷, we have discovered a pervasive influence of hybridization throughout their evolutionary histories. Gene exchange among distinctive forms has contributed to existing diversity, supporting the hypothesis that introgressive hybridization can play a significant role in evolution of vertebrates.

Cyprinid fishes of the genus *Gila* have attracted considerable attention because of their unique morphological adaptations to diverse riverine habitats⁸. Three morphologically distinctive species were broadly sympatric throughout the Colorado River basin. *Gila cypha* once occupied canyon-bound reaches of the middle and upper basin, whereas *G. elegans* formerly occupied most of the basin's largest rivers. Both possess slender, streamlined bodies with leathery skins, embedded scales, and falcate fins, characteristics well suited for surviving in abrasive, high-gradient, flood-prone rivers. A third species, *G. robusta*, exhibits a more generalized morphology and lives in large and small rivers throughout the basin.

Morphological analyses⁸⁻¹⁰ indicated that *G. cypha*, *elegans* and *robusta* maintain their distinctiveness in sympatry, with phenotypically intermediate individuals occasionally noted^{11,12}. In addition, geographically isolated stocks such as *G. seminuda* (until recently a subspecies of *G. robusta*) were found to be morphologically intermediate to these three species, prompting speculation of hybrid origin⁸. Molecular and morphological examination of *G. seminuda* supported its hypothesized hybrid origin, as it exhibited a mosaic of allozymic and mitochondrial DNA characteristics otherwise unique to *G. elegans* or *robusta*¹³.

To further study the potential significance of introgression among Colorado River *Gila*, we examined allozymic and mtDNA variation within and among taxa. Morphologically identified *G. cypha*, *elegans* and *robusta* from now-allopatric populations exhibited unique allozymic and mtDNA characteristics (Table 1), identifying them as distinct phylogenetic lineages. Despite this evolutionary independence, local introgression among all three taxa has clearly occurred in the past. In the upper Colorado River basin where *G. robusta* and *cypha* now coexist, morphologically identified *G. robusta* exhibited allozyme characters of both species and mtDNA typical of *G. cypha* (Table 1). Isolates (such as *G. seminuda*, from the Virgin River; *G. robusta jordani* from the pluvial White River, a now-isolated tributary to the Virgin River; and *G. robusta* from the upper Little Colorado River) also possessed derived allozyme alleles of all three species (Table 1) and *G. cypha* or *elegans* mtDNA (Table 1). Neither *G. cypha* nor *elegans* have been recorded from the Virgin, pluvial White, or upper Little Colorado rivers, with the last two systems isolated by barriers at least since mid-Holocene¹⁴ and late Pleistocene¹⁵, respectively.

Hybridization was further examined by contrasting phylogenies inferred from allozymes and mtDNA¹⁶. *Gila atraria*, native to the adjacent Bonneville basin¹⁷, was included because of similarity of its mtDNA to that of Colorado basin *Gila* (T.E.D., unpublished data). *Gila bicolor*, a distant relative¹⁸, was included as an outgroup. Phenetic and cladistic analyses produced similar topologies within character sets, but results were discordant between sets (Fig. 1). With allozyme data (Fig. 1a, b), Colorado basin *Gila* were monophyletic. Where hybridization was suspected and all tissues available, populations were phenetically intermediate to *G. robusta* and *cypha*. The distantly related *G. atraria* and *bicolor* comprised independent clusters/clades, neither similar to Colorado River forms. Topologies based on mtDNA characters (Fig. 1c, d) were in sharp contrast. Although populations of *G. robusta* from the lower Colorado basin were distinct, *robusta* from the Little Colorado and Green rivers and *G. robusta jordani* had mtDNA similar to that of *cypha*, and *G. seminuda* exhibited mtDNA almost identical to *G. elegans*. Most notably, Colorado basin *Gila* were not monophyletic, as *G. cypha* mtDNA was more closely related to that of *G. atraria* (especially from the Sevier River) than it was to either *G. elegans* or *robusta* mtDNAs.

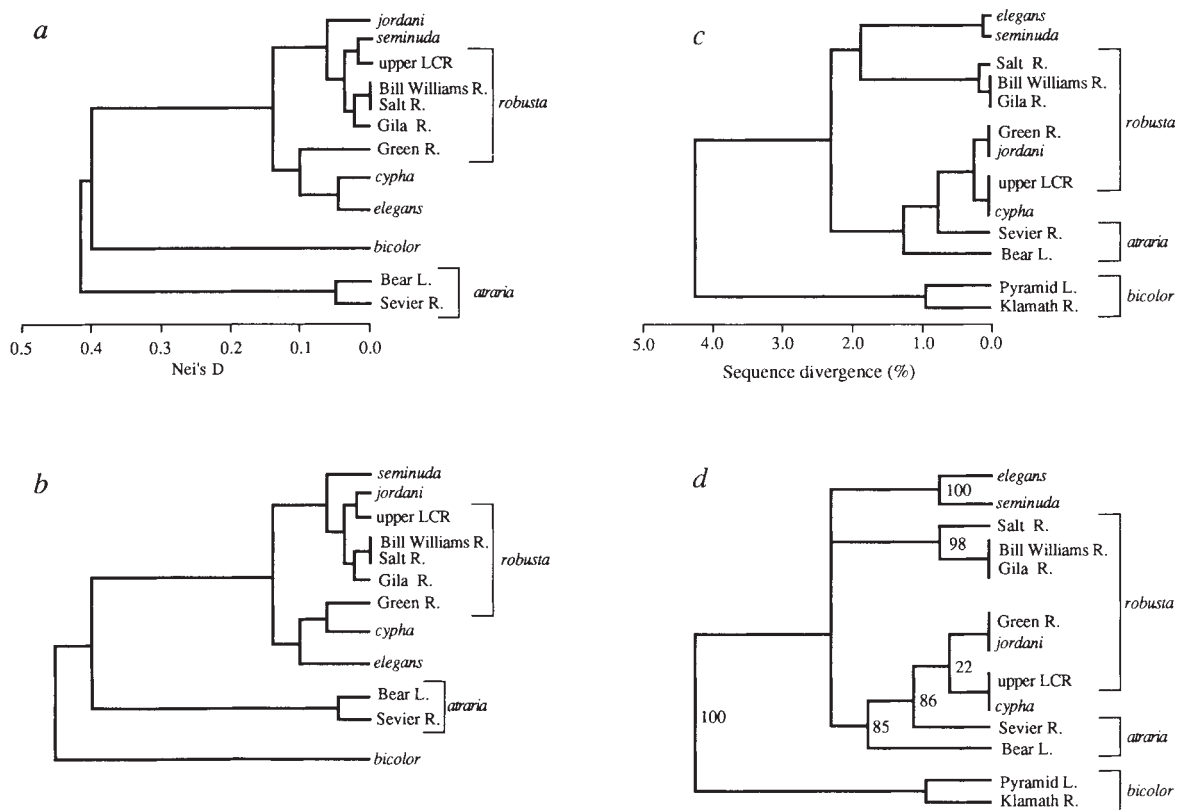


FIG. 1 Analysis of evolutionary relationships among *Gila*. **a**, UPGMA topology³⁰ based on Nei's distances³¹ calculated from 28 presumptive allozyme loci: *mAH-A*, *sAH-A*, *AK-A*, *ADH-A*, *mAAT-A*, *sAAT-A*, *CBP-1*, *CBP-2*, *CK-A*, *FH-A*, *GP-1*, *G3PDH-A*, *GPI-B*, *mIDHP-A*, *sIDHP-A*, *LDH-A*, *LDH-B*, *LDH-C*, *mMDH-A*, *sMDH-A*, *sMDH-B*, *mMEP-A*, *PEPA*, *PEPB*, *PEPD*, *PEPS*, *PGM-A*, and *sSOD-A*. Conditions for resolution described in refs 13 and 32. **b**, Most parsimonious tree (59.3 steps) resolved from allozyme allele frequencies using FREQPARS³³. Alternative phylogenetic topologies were evaluated by comparing total tree lengths. **c**, UPGMA topology³⁴ based on pairwise estimates of sequence divergence³⁵ from mtDNA restriction sites as described in refs 13 and 36. Each mtDNA sample was characterized with the following restriction endonucleases: *Bam*HI, *Bcl*I, *Bgl*II, *Bst*EI, *Eco*RI, *Hind*III, *Mlu*I, *Nco*I, *Nde*I, *Nhe*I, *Pvu*II, *Sac*I, *Xba*I and *Xho*I. **d**, Strict consensus of five most parsimonious trees (62 steps, consistency index=0.74, retention index=0.81) resolved from a branch and bound analysis of presence/absence of mtDNA restriction sites using PAUP³⁷. Numbers on the nodes represent proportion of 1,000 bootstrap replicates in which the particular node was

monophyletic in the 50% majority rule consensus tree.

METHODS. Samples for phylogenetic analysis were obtained from the following locations (sample sizes for allozymes and mtDNA respectively): *G. atraria*, Sevier R., Piute Co., UT (18, 2) and Spring Cr (Bear Lk. drainage) Bear Lake Co., ID (15, 3); *G. bicolor*, Pyramid Lk., Washoe Co., NV (10, 3) and Sprague R. (Klamath R. drainage), Klamath Co., OR (0, 1); *G. cypha*, lower Little Colorado R., Coconino Co., AZ (20, 5); *G. elegans*, Lk. Mohave, Mohave Co., AZ (20, 4—from captive stock at Dexter National Fish Hatchery and Technology Center, Dexter, NM¹³); *G. robusta jordanii*, pluvial White R. drainage, Lincoln Co., NV (20, 4—from captive stock at Dexter National Fish Hatchery and Technology Center, Dexter, NM); *G. robusta*, Burro Cr. (Bill Williams R. drainage), Yavapai Co., AZ (30, 4), Aravaipa Cr. (Gila R. drainage), Graham Co. (17, 2), and Cherry Cr. (Salt R. drainage), Gila Co. (20, 6), Green R., Moffat Co., UT (20, 7), and Chevelon Cr. (upper Little Colorado R. drainage), Navajo Co., AZ (21, 3); *G. seminuda*, Virgin R., Mohave Co., AZ (17, 4). Samples of *robusta* used only for analysis of introgression (Table 1) were from: Colorado R. near Rifle, Garfield Co., Debeque Canyon, Mesa Co., CO and Gunnison R., Delta Co., CO.

Relationships provided by allozymes were consistent with predictions from morphology^{18,19} and geography²⁰. Therefore, relationships based on mtDNA probably do not reflect organismal phylogeny, with the similarity of *G. atraria* and *cypha* mtDNA being especially problematic. Discordance could result from hybridization¹⁶, stochastic inheritance of ancestral polymorphisms^{21,22} or homoplasy²³, with the last two typified by poor phylogenetic resolution^{21,23}. Bootstrap analysis²⁴, however, indicated the mtDNA phylogeny was well resolved; the node supporting monophyly of *G. atraria* and *cypha* was in 85% of 1,000 replicates (Fig. 1d). Additionally, forcing *G. atraria* outside a clade composed of *cypha*, *elegans* and *robusta* (as indicated by allozymic and morphological data) produced a mtDNA-based tree of 71 steps, 9 (15%) longer than the most parsimonious trees (Fig. 1d). For allozyme data, forcing *G. cypha* and *atraria* into a sister-group relationship requires 82.2 steps, 22.9 (39%) longer than the most parsimonious allozyme-based tree. Therefore, discordance of mtDNA and allozyme topologies was most probably due to introgression of mtDNA among species.

Similarity of mtDNAs from isolates (such as *G. robusta jordanii*, *seminuda*, upper Little Colorado River *robusta*) with

mtDNAs of *G. cypha* and *elegans* indicated introgression occurred relatively recently. Ever-increasing aridity during Pleistocene²⁵ and Holocene²⁶ almost certainly altered aquatic habitats dramatically and may have promoted hybridization to produce existing mosaics. The greater divergence between *G. cypha* and *atraria* mtDNAs indicates a far earlier hybridization event. Recent introgression between *atraria* and *cypha* would also be reflected in nuclear genes, yet diagnostic allozymes failed to provide such evidence (Table 1). Therefore, phylogenetic similarity must have resulted from an old episode of introgression, reflecting ancestral drainage connection between the now-independent Bonneville and Colorado basins.

Gila is the most morphologically diverse genus of minnows in western North America. Our results support the hypothesis that introgressive hybridization has played a significant role in generating this diversity¹³ by providing additional genetic variation for selection and drift to mould into locally distinctive phenotypes^{2,8}. Thus, Colorado River *Gila* represent a complex of self-maintaining, genetically distinctive species which are capable of exchanging genetic material (comparable to syngameons in plants⁷). Botanists recognize the importance of introgressive hybridization in evolution^{27,28}. Our results for *Gila*

TABLE 1 Frequencies of diagnostic alleles

Locus	Allele	Allopatric populations				Isolated populations			Sympatric populations		
		<i>G. atraria</i>	<i>G. cypha</i>	<i>G. elegans</i>	<i>G. robusta</i>	<i>G. seminuda</i>	<i>G. jordani</i>	upper LCR	Green R.	upper	Colorado R.
M-Aat-A	a	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
	b	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		1.00
		(33)	(20)	(20)	(69)	(43)	(20)	(21)	(40)		(55)
Ak-A	a	0.00	0.55	0.00	0.00	0.32	0.50	0.00	0.26		0.73
	b	1.00	0.20	1.00	1.00	0.67	0.50	1.00	0.36		0.24
	c	0.00	0.10	0.00	0.00	0.01	0.00	0.00	0.35		0.03
	d	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.03		0.00
		(33)	(20)	(20)	(69)	(38)	(20)	(21)	(50)		(55)
Cbp-1	a	1.00	0.25	0.00	0.96	0.43	1.00	1.00	0.00		0.00
	b	0.00	0.75	1.00	0.04	0.57	0.00	0.00	1.00		1.00
		(33)	(20)	(20)	(69)	(43)	(20)	(21)	(50)		(55)
Ck-A	a	1.00	0.33	0.00	1.00	0.51	1.00	0.90	0.44		0.37
	b	0.00	0.67	1.00	0.00	0.49	0.00	0.10	0.56		0.63
		(33)	(20)	(20)	(69)	(43)	(20)	(21)	(50)		(55)
Pgm-A	a	0.00	0.00	0.58	0.29	0.20	0.00	0.00	0.00		0.02
	b	0.02	1.00	0.48	0.71	0.80	1.00	1.00	1.00		0.98
	c	0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
		(33)	(20)	(20)	(69)	(43)	(20)	(21)	(45)		(55)
mtDNA		'atraria'	'cypha'	'elegans'	'robusta'	'elegans'	'cypha'	'cypha'	'cypha'		'cypha'
		(5)	(5)	(4)	(16)	(24)	(4)	(3)	(7)		(2)

Sample sizes (in parentheses) and distribution of diagnostic allozyme alleles and mtDNA haplotypes from *Gila* populations examined for evidence of introgression. Diagnostic allozyme markers were discovered in a survey of 28 presumptive loci (described in Fig. 1). Alleles are designated by ascending mobility, with 'a' most cathodal; locus nomenclature follows ref. 29. mtDNA haplotypes were identified by digestion with a minimum of two restriction enzymes found diagnostic for these taxa. Locality information provided in Fig. 1. Some categories are represented by several populations: 'allopatric *G. atraria*' were obtained from the Sevier and Bear Lake drainages; 'allopatric *G. robusta*' were sampled from the Bill Williams, Gila, Salt and Verde rivers; sympatric populations from the 'Green R.' sample were morphologically identified *G. robusta* from the Green, Yampa and Little Snake rivers while 'upper Colorado R.' represents similar specimens from Gunnison River and DeBeque Canyon and Rifle areas of the Colorado River; and *G. seminuda* are from the Virgin and Moapa rivers.

indicate that zoologists must do the same. In addition, hybridization among these critically endangered taxa has been considered primarily as a detrimental, anthropogenic phenomenon⁸, but the apparently pervasive influence of natural introgression suggests that conservation strategies should evaluate the impact of hybridization in a new light. □

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- Anderson, E. *Introgressive Hybridization* (Wiley, New York, 1941).
- Grant, V. *Plant Speciation* 2nd edn (Columbia Univ. Press, New York, 1981).
- Harrison, R. G. in *Oxford Surveys in Evolutionary Biology* Vol. 7 (eds Futuyma, D. & Antonovics, J.) 69–128 (Oxford University Press, New York, 1990).
- Mayr, E. 1963. *Animal Species and Evolution* (Belknap, Harvard University Press, Cambridge, 1963).
- Lewontin, R. C. & Birch, L. C. *Evolution* **20**, 315–336 (1966).
- Short, L. L. *Ann. Mo. Bot. Gdn* **59**, 447–453 (1972).
- Lee, D. S. et al. (eds) *Atlas of North American Freshwater Fishes* (NC State Mus. Nat. Hist., Raleigh, 1980).
- Smith, G. R., Miller, R. R. & Sable, W. D. *Proc. First Conf. Sci. Res. Nat. Parks* **1**, 613–623 (1979).
- Rinne, J. N. *Wasmann J. Biol.* **34**, 65–107 (1976).
- Douglas, M. E., Minckley, W. L. & Tyus, H. M. *Copeia* **1989**, 653–662 (1989).
- Smith, G. R. *Trans. Bonneville Chapter Am. Fish. Soc.* **1983**, 1–8 (1983).
- Holden, P. B. & Stalnaker, C. B. *Copeia* **1970**, 409–420 (1970).
- DeMarais, B. D., Dowling, T. E., Douglas, M. E., Minckley, W. L. & Marsh, P. C. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2747–2752 (1992).
- Hubbs, C. L. & Miller, R. R. *Bull. Univ. Utah* **38**, Biol. Ser. **10**, 17–144 (1948).
- Wolfe, E. W. in *Landscapes of Arizona* (eds Smiley, T., Nations, J., Péwé, T. & Schafer, J.) 111–136 (University Press America, New York, 1984).
- Smith, G. R. *Syst. Biol.* **41**, 41–57 (1992).
- Sigler, W. F. & Miller, R. R. *Fishes of Utah* (UT St. Dept. Fish & Game, Salt Lake City, 1963).
- Hubbs, C. L., Miller, R. R. & Hubbs, L. C. *Mem. Calif. Acad. Sci.* **7**, 1–259 (1974).
- Miller, R. R. *Copeia* **1945**, 104–110 (1945).
- Minckley, W. L., Hendrickson, D. A. & Bond, C. E. in *The Zoogeography of North American Freshwater Fishes* (eds Hocutt, C. H. & Wiley, E. O.) 519–613 (Wiley, New York, 1986).
- Neigel, J. E. & Avise, J. C. in *Evolutionary Processes and Theory* (eds Nevo, E. & Karlin, S.) 515–534 (Academic, New York, 1986).
- Pamilo, P. & Nei, M. *Molec. Biol. Evol.* **5**, 568–583 (1988).
- Dowling, T. E. & Brown, W. M. *Syst. Zool.* **38**, 126–143 (1989).
- Felsenstein, J. *Evolution* **39**, 783–791 (1985).
- Winograd, I. J. et al. *Science* **258**, 255–260 (1992).
- Axelrod, D. I. *Calif. Acad. Sci. Occ. Pap.* **132**, 1–74 (1979).
- Arnold, M. L. A. *Rev. ecol. Syst.* **23**, 237–261 (1992).
- Rieseberg, L. H. & Brunsfeld, S. in *Plant Molecular Systematics* (eds Soltis, D. E., Soltis, P. S. & Doyle, J. J.) 151–176 (Chapman and Hall, New York, 1992).
- Shaklee, J. B., Allendorf, F. W., Moritz, D. C. & Whitt, G. S. *Trans. Am. Fish. Soc.* **119**, 2–15 (1990).
- Swofford, D. L. & Selander, R. B. *J. Hered.* **72**, 281–283 (1981).

- Nei, M. *Genetics* **89**, 583–590 (1978).
- Murphy, R. M., Sites, J. W. Jr, Butth, D. G. & Haufier, C. H. in *Molecular Systematics* (eds Hillis, D. M. & Moritz, C.) 45–126 (Sinauer, Sunderland, MA, 1990).
- Swofford, D. & Berlocher, S. H. *Syst. Zool.* **36**, 293–325 (1987).
- Nei, M., Stephens, J. C. & Saitou, N. *Molec. Biol. Evol.* **2**, 66–85 (1985).
- Nei, M. & Tajima, F. *Genetics* **105**, 207–217 (1983).
- Dowling, T. E., Moritz, C. & Palmer, J. D. in *Molecular Systematics* (eds Hillis, D. M. & Moritz, C.) 250–317 (Sinauer, Sunderland, MA, 1990).
- Swofford, D. L. *Phylogenetic Analysis Using Parsimony, version 3.0q* (IL Nat. Hist. Surv., Champaign, 1990).

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Agrobacterium conjugation and gene regulation by N-acyl-L-homoserine lactones

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CONJUGAL opines secreted by crown gall tumours induce strains of *Agrobacterium tumefaciens* that are donors of Ti plasmids to produce a diffusible conjugation factor¹. This enhances the conjugal transfer efficiency of the Ti plasmid in other strains of *A. tumefaciens*. This factor behaves as a secondary messenger,

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transmitting the environmental information to *tra* genes. Here we report the use of spectrometry to show that this factor is identical to synthetic *N*-(β -oxo-octan-1-oyl)-L-homoserine lactone and confirm that the synthetic compound is biologically active. *N*-(Hexan-1-oyl)-L-homoserine lactone has also been detected. A closely related molecule, *N*-(β -oxo-hexan-1-oyl)-L-homoserine lactone, autoinduces bioluminescence in the distantly related bacterium, *Vibrio fischeri*². *N*-Acyl-homoserine lactones thus seem to be conserved molecules in which the length and nature of the lipophilic acyl chain determines the biological function to be regulated. Mutants that do not produce the factor fail to conjugate unless supplied with it in the induction medium (our unpublished data). These data indicate that the conjugation factor is an autoinducer and a key signal molecule in the conjugation system of *A. tumefaciens*. It is, to our knowledge, the first example of a second messenger molecule in a bacterial conjugation system.

Conjugation factor (CF) is present in the culture supernatant of transfer constitutive strains of *A. tumefaciens*¹. Its purification using high-performance liquid chromatography (HPLC) yielded two active components, CF1 and CF2. Examination of these fractions by chemical ionization quadrupole mass spectrometry showed strong quasimolecular (*M*+*H*) ions, with an *m/z* of 242 for CF1, and for the less homogeneous CF2 fraction, ions at *m/z* 274, 214 and 200. All of these even *M*+*H* ions were consistent with the presence of nitrogenous components. For CF1 this was confirmed by infrared spectrometry, which showed amide I and II carbonyl absorptions at 1,646 and 1,540 cm^{-1} as well as characteristic absorptions near 1,780 cm^{-1} and 1,714 cm^{-1} assignable to the presence of γ lactone and ketonic carbonyls². Acid hydrolysis of CF1 yielded an amino acid which comigrated with homoserine when separated by high-voltage paper electrophoresis. Daughter ion tandem mass spectrometry of the *M*+*H* ions at 242 *m/z* in CF1, and *M*+*H* ions at 274, 214 and 200 in CF2 fraction showed related fragmentations after helium collisional activation. Except for the 214 parent ion, which showed a daughter ion at 113 instead of 99 as well as ions at 102 and 141, each daughter ion set from the parent ions at 242, 274 and 200 *m/z*, displayed prominent 99, 102 and 141 *m/z*

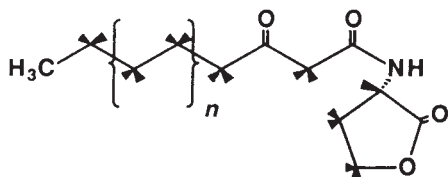


FIG. 1 Structural formulae for *N*- β -oxo-acyl-amides of L-homoserine lactone, *n*=0, 1 and 2 correspond to the *N*- β -oxo-hexanoyl-, -octanoyl and -decanoyl amides. For isolation of the conjugation factor CF1 (*n*=1), strain K794, a transfer constitutive octopine strain of *A. tumefaciens*, was grown in Petit's liquid minimal medium⁷ for 2 days at 28 °C, using proline and mannitol as the nitrogen and carbon sources. Presence of CF activity was determined by induction of a *Tra::lacZ749* reporter gene fusion in strain NT-1 (supplied by S. Farrand³). CF was extracted from K794 bacterial culture filtrates by ethyl acetate. Partial purification of the concentrated extracts was achieved by flash chromatography on silica gel using ethyl acetate as eluent⁸, followed by C18 reversed-phase open column flash chromatography eluted isocratically with 30% (v/v) methanol/water. Evaporation of the active fractions gave a yellowish white solid (3 mg L⁻¹ culture). Further purification by C18 reversed-phase HPLC (analytical 25 cm, 4 mm inner diameter) eluted (1 mL min⁻¹) with a linear gradient of 30–45% methanol, gave two separate biologically active components, labelled CF1 (retention time 13.38 min) and CF2 (retention time 11.4 min). A quantitative β -galactosidase assay⁹, was used to measure CF activity. Synthetic CF1 (*n*=1) was obtained in 49% yield in a one-step procedure using L-homoserine lactone HCl (1 mmol) in pyridine (10 ml) at 130 °C for 45 min with the acyl Meldrum's acid intermediate of Oikawa¹⁰ (1.5 mmol). Cognate preparations proceeded smoothly with the appropriate intermediate¹⁰. Saturated acyl amides were prepared from the L-homoserine lactone HCl and corresponding acyl chloride in pyridine under the same conditions.

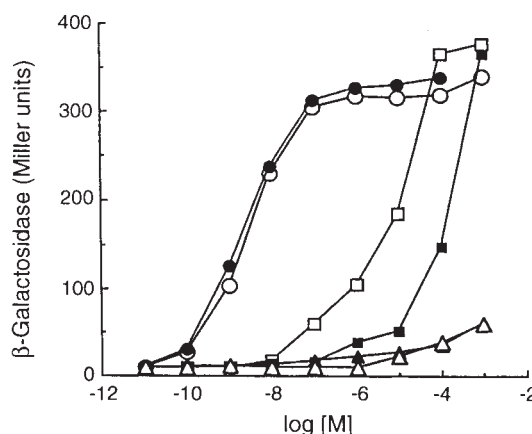


FIG. 2 Selective induction of *tra* gene activity by *N*-acyl-amides of L-homoserine lactone. Natural: CF1 (●); synthetic: *N*- β -oxo-octanoyl- (○), *N*- β -oxo-hexanoyl- (□), *N*- β -oxo-decanoyl- (■), *N*-octanoyl- (△) and *N*-hexanoyl-amides (▲) of L-homoserine lactone. β -Galactosidase activity was expressed as Miller units⁹. *N*-acyl-amides of L-homoserine lactone were added to the minimal medium⁷ plus (NH₄)₂SO₄ (0.2, w/v) and mannitol (0.2%, w/v) and then inoculated with a 1:40 dilution of a late log phase culture (absorbance at 620 nm $\approx$ 0.6) of NT-1 (*traR*, *tra::lacZ749*)³. After 22 h of incubation at 25 °C, cells were collected by centrifugation and assayed for β -galactosidase activity¹¹.

ions. Comparison of the corresponding electron impact data with those reported² suggested a closely related set of homoserine lactone derivatives. These data taken together with the reported³ homology of TraR in *A. tumefaciens* with LuxR in *Vibrio fischeri*, which requires *N*-(β -oxo-hexan-1-oyl)-L-homoserine lactone for gene induction, indicate that a common homoserine lactone moiety is associated with gene regulation in these distantly related species.

The known luciferase autoinducer β -oxo-hexanol homoserine of *V. fischeri*² (Fig. 1, structure *n* = 0) was synthesized to compare its mass spectrum with that of natural CF. The comparison confirmed its relatedness, but nonidentity with the tandem ion mass spectrometry daughter ion spectra from the 214 *m/z* parent ion in the CF2 fraction. But data from the homogeneous CF1 fraction were consistent with a $-(\text{CH}_2)_2-$ extension of the lipophilic side chain of the luciferase autoinducer, i.e. β -oxo-octanoyl homoserine lactone (Fig. 1, *n* = 1). This was also synthesized. These molecules have very similar infrared spectra with the four characteristic carbonyl signals². In the case of CF1, sufficient homogeneous material was obtained to show that all the measured properties (ultraviolet, infrared, ¹H nuclear magnetic resonance (NMR), ¹³C NMR; chromatographic parameters data not shown) and biological activity (Fig. 2) were virtually indistinguishable from those of the synthetic sample. The tandem mass spectrometric fragments of the *M*+*H* ion of *m/z* 200 present in the CF2 fraction suggested it was the fully reduced hexanoyl amide of homoserine lactone and the daughter ion fragmentation patterns were indistinguishable from those of a synthetic sample of the hexanoyl amide.

Figure 2 shows *tra* gene activity induced by the natural CF1 from *A. tumefaciens* and by synthetic *N*- β -oxo-octanoyl-, *N*- β -oxo-hexanoyl-, *N*- β -oxo-decanoyl-, *N*-octanoyl- and *N*-hexanoyl amides of L-homoserine lactone. Only the synthetic *N*- β -oxo-octanoyl-L-homoserine lactone shows identity in gene induction activity with the natural product and accounts fully for the observed activity of fraction CF1. Although masses corresponding to the *N*-hexanoyl amide were detected by mass spectrometry in the biologically active CF2 fraction from the HPLC fractionation, the synthetic product had insufficient activity on a molar basis to account for the (autoinducing) activity of this fraction. The close relationship of the daughter ions from the 274 parent ion to those of synthetic conjugation

factor m/z 242 and in particular the presence of a strong fragment ion at 134 instead of the ion corresponding to a protonated homoserine lactone at 102, suggested that this fraction contained the methyl ester of the conjugation factor derived as a methanolic-solvent transesterification artefact. This was confirmed by the chromatographic behaviour, CI daughter ion mass spectra, infrared spectra and biological activity of the synthetic acyclic methyl ester of CF1.

The remarkable feature of the data in Fig. 2 is that extension (Fig. 1, $n = 2$) or diminution (Fig. 1, $n = 0$) of the chain length or conversion of the beta keto groups (Fig. 1, $n = 0$ and $n = 1$) to methylene groups is sufficient to reduce the inducing activity by four orders of magnitude or more compared to that of the β -oxo-octanoyl derivative ($n = 1$). A similar specificity was reported for analogues of the autoinducer of bioluminescence in *V. fischeri*⁴.

The facts that N - β -oxo-hexanoyl-homoserine lactone is the autoinducer for luminescence activity in *V. fischeri*² and for

antibiotic biosynthesis in *Erwinia*⁵, that N - β -hydroxy-butanoyl-L-homoserine lactone is the autoinducer for luminescence in *V. harveyi*⁶ and that N - β -oxo-octanoyl-L-homoserine lactone is the autoinducer for conjugation in *A. tumefaciens*, suggest that N -acyl homoserine lactones may be a widely conserved signal for gene regulation. □

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1. Zhang, L. & Kerr, A. *J. Bact.* **173**, 1867–1872 (1991).
2. Eberhard, A. *et al. Biochemistry* **20**, 2444–2449 (1981).
3. Piper, K. R., Beck von Bodman, S. & Farrand, S. K. *Nature* **362**, 448–450 (1993).
4. Eberhard, A., Widrig, C. A., McBath, P. & Schineller, J. B. *Arch. Microbiol.* **146**, 35–40 (1986).
5. Bainton, N. J. *et al. Gene* **116**, 87–91 (1992).
6. Cao, J.-G. & Meighen, E. A. *J. biol. Chem.* **264**, 21670–21676 (1989).
7. Petit, A. & Tempé, J. *Molec. gen. Genet.* **167**, 147–155 (1978).
8. Still, W. C., Kahn, M. & Mitra, A. *J. org. Chem.* **43**, 2923–2925 (1978).
9. Miller, J. H. *Experiments in Molecular Genetics* 352–359 (Cold Spring Harbor Laboratory Press, New York, 1972).
10. Oikawa, Y., Sugano, K. & Yonemitsu, O. *J. org. Chem.* **43**, 2087–2088 (1978).
11. Stachel, S. E., An, G., Flores, C. & Nester, E. W. *EMBO J.* **4**, 891–898 (1985).

Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction

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CONJUGAL transfer of Ti plasmids from *Agrobacterium* donors to bacterial recipients is controlled by two types of diffusible signal molecules. Induction is mediated by novel compounds, called opines, that are secreted by crown gall tumours. These neoplasias result from infection of susceptible plants by virulent *agrobacteria*. The second diffusible signal, called conjugation factor, is synthesized by the donor bacteria themselves. Production of this factor is induced by the opine. Here we show that conjugation is regulated directly by a transcriptional activator, TraR, which requires conjugation factor as a coinducer to activate *tra* gene expression. TraR is a homologue of LuxR, the *lux* gene activator from *Vibrio fischeri* which also requires an endogenously synthesized diffusible coinducer. The two regulatory systems are related; the two activator proteins show amino-acid sequence similarities and the *lux* system cofactor, autoinducer, will substitute for conjugation factor in the TraR-dependent activation of Ti plasmid *tra* genes.

Agrobacterium tumefaciens strains harbouring pTiC58 induce plant tumours that synthesize the opines agrocinopines A and B^{1,2}. Either opine induces a Ti plasmid-encoded system required for their catabolism by the bacterium, and also a conjugation system that transfers the Ti plasmid to bacterial recipients^{3,4}. Coordinate regulation of these systems operates through a single transcriptional repressor, AccR, encoded by the *accR* gene⁵. A Ti plasmid with a deletion in *accR* constitutively expresses both phenotypes. But two lines of evidence indicate that conjugation is regulated at a second level. First, a Tn5 insertion mutant of pTiC58, 10-23, is constitutive for conjugal transfer (Tra^c), but remains inducible for agrocinopine catabolism⁶. The 10-23 insertion is not located in *accR* but maps to *EcoRI* fragment 33 in a region encoding conjugal transfer functions (Fig. 1). Furthermore, the mutation is dominant suggesting that it results in a

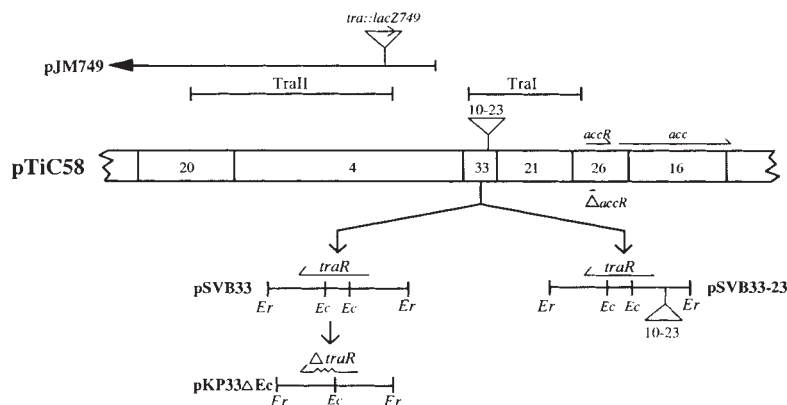
gain of function⁶. Second, conjugation requires a diffusible substance called conjugation factor (CF)⁷. This signal, produced by the donor population itself, accumulates in culture supernatants after induction by the opines. *Agrobacterium* donors that do not produce CF on induction with opines fail to conjugate unless exposed to the factor produced by a CF⁺ strain⁷.

To examine this second level of regulation, we tested *Agrobacterium* strains with or without Ti plasmids for their ability to express the *tra::lacZ749* reporter fusion carried on pJM749 (Fig. 1). This clone lacks *accR* and also the region defined by the 10-23 mutation. Strain NT1(pJM749), which lacks a Ti plasmid and therefore the *accR* gene, did not produce β -galactosidase (Table 1). But strains harbouring the $\Delta accR$ or the 10-23 Tra^c Ti plasmids expressed the *lacZ* fusion at high levels. Failure of the Ti plasmidless strain to express the reporter fusion indicates that the *tra* genes are positively regulated. Expression in the 10-23 mutant suggested that the Tn5 insertion promotes expression of the regulatory gene, we call *traR*, responsible for *tra* gene activation. To test this, we cloned the Tn5-containing *EcoRI* fragment 33 from mutant 10-23 to form pSVB33-23 (Fig. 1). In two experiments, a donor strain harbouring this plasmid *in trans* to pTiC58 transferred the Ti plasmid by conjugation to the *A. tumefaciens* recipient C58C1RS⁶ at frequencies of 4.8×10^{-2} and 6.7×10^{-2} per input donor without induction by agrocinopines. These values are similar to transfer frequencies observed for the $\Delta accR$ and the 10-23 Tra^c mutant Ti plasmids⁶. In the absence of pSVB33-23, transfer of pTiC58 was undetectable ($<10^{-8}$ per input donor). We conclude that *traR*, expressed from pSVB33-23, activates conjugal transfer of pTiC58 in the absence of the inducer opine.

Because *traR* on *EcoRI* fragment 33 *trans*-activates conjugation, we determined if this fragment is sufficient to induce expression of the *tra::lacZ749* fusion on pJM749 in the absence of a Ti plasmid. Plasmids pSVB33-23, and pSVB33 which contains *EcoRI* fragment 33 from wild-type pTiC58 (Fig. 1), were introduced into strain NT1(pJM749). Neither strain produced significant levels of β -galactosidase (Table 1). But unlike the pJM749 merodiploids harbouring Tra^c Ti plasmids, these strains do not produce conjugation factor. When the two reporter strains were incubated with a crude preparation of CF, β -galactosidase levels were elevated 400-fold (Table 1). This response required pSVB33 or pSVB33-23. The *tra::lacZ* reporter fusion was not expressed when the cultures were supplemented with a preparation from a strain that does not produce CF (data not shown). Zhang *et al.*⁸ show in the accompanying letter that purified CF stimulates TraR-dependent expression of the reporter fusion in a manner indistinguishable from that observed with our crude preparation. These results indicate that the *trans*-acting

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FIG. 1 Physical map of the conjugal transfer-opine catabolism region of pTiC58. The map shows the locations of *EcoRI* sites between plasmid kilobase coordinates 111 and 134, the limits of *Tra* regions I and II (ref. 6), and the recombinant clones used in this work. The locations of genes encoding catabolism of agrocinosines A and B (*acc*)⁴ and the master conjugal transfer-opine catabolism repressor (*accR*)⁵ are shown above the map. The small line labelled $\Delta accR$ shows the location of the 5 bp deletion in *accR* responsible for the *Tra*^c/*Acc*^c phenotype of the spontaneous mutant plasmid, pTiC58 $\Delta accR$. The triangle labelled 10-23 depicts the site of the Tn5 insertion in wild-type pTiC58 that results in a *Tra*^c/*Acc*-inducible phenotype⁵. pJM749, containing the *tra::lacZ749* reporter fusion, was derived by Tn3HoHo1 mutagenesis²² of pTHB58, a cosmid clone containing an insert from pTiC58⁴. When marker-exchanged into pTiC58 $\Delta accR$ the *lacZ749* insertion results in a *Tra*⁻ phenotype⁵. The direction of transcription of the *lacZ* fusion is indicated by the arrowhead. pSVB33 and pSVB33-23 were produced by cloning *EcoRI* (*Er*) fragment 33 from pTiC58 and pTiC58Tra^c10-23, respectively, into the IncW vector pSa152 (ref. 23). The location and transcriptional direction of the *traR* ORF is indicated for the two plasmids. pKP33 ΔEc was produced by deleting a 121 bp *EclXI*



(*Ec*) fragment internal to *traR* in pSVB33. The allele is labelled $\Delta traR$ with the wavy line indicating that portion of the *TraR* amino-acid sequence altered by the internal deletion.

function encoded by *traR* requires CF to activate expression of *tra* genes.

Addition of the CF preparation to strain C58 harbouring pJM749 did not stimulate expression of the *tra::lacZ* fusion (Table 1). This is consistent with the observation that CF will not replace opines for induction of transfer.⁷ That the reporter fusion is expressed in strains harbouring the $\Delta accR$ *Tra*^c mutant Ti plasmid but not in wild-type strain C58, even when exposed to exogenous CF, indicates that expression of *traR* is regulated by the opines through *AccR*.

EcoRI fragment 33 was sequenced by the dideoxynucleotide chain termination method⁹ (EMBL accession no. Z15003). The 1,805 base pair (bp) fragment contains a 702 bp open reading frame (ORF) between an ATG at bp 1,244 and TGA at bp 542. This corresponds to an anticlockwise transcriptional orientation on the standard map of pTiC58 (ref. 10). The 10-23 Tn5 insertion is located between bp 1,482 and 1,483, 238 bp upstream of the ATG initiation codon. This location is consistent with our hypothesis that the *Tra*^c phenotype results from promoted expression of the ORF by the transposon. Dependence on the intact ORF was determined by deleting an internal 120 bp *EclXI* fragment (Fig. 1). Assuming that the ORF represents *traR* and encodes a protein, the deletion removes amino acids 107-146 and alters the remaining sequence of the protein. This clone, pKP33 ΔEc , does not activate β -galactosidase expression from the *tra::lacZ749* reporter fusion even in the presence of CF (Table 1).

The *traR* ORF can encode a protein of 234 amino acids with an *M_r* of 26,506. The protein, we name *TraR*, exhibits 20% amino-acid identity and 62% amino-acid similarity over a 234 residue overlap with the bacterial protein *LuxR* (Fig. 2). *LuxR* is a transcriptional activator that regulates bioluminescence gene expression in the marine bacterium, *Vibrio fischeri*¹¹. Activation of *lux* expression also requires a diffusible cofactor called autoinducer (AI)¹². This factor is produced by *V. fischeri* cells themselves and, along with *LuxR*, regulates bioluminescence by a mechanism called autoinduction¹³. Given the similarity between *TraR* and *LuxR*, we determined whether AI can substitute for CF in the *TraR*-dependent activation of the *tra::lacZ749* fusion on pJM749. Cells incubated with a preparation containing AI produced levels of β -galactosidase activity 40-fold higher than those in cells receiving no additions (Table 1). But these levels were 10-fold lower than those obtained with preparations containing CF.

These results show that conjugal transfer of the *Agrobacterium* plasmid pTiC58 is controlled by autoinduction. CF clearly functions as a second message to transmit the opine signal to the

conjugation regulatory apparatus. Furthermore, CF is produced by *Agrobacterium* strains harbouring any one of several Ti plasmid types⁷ indicating that this mode of regulation is common to the *tra* systems of these plasmids. CF may, as with AI, serve to stimulate donors in a population-dependent manner. Alternatively, because conjugation is induced by opines, CF may function to amplify the response produced by suboptimal concentrations of these signal molecules. Of more general significance, the system is remarkably similar to that which controls bioluminescence in *V. fischeri*. The activators, *LuxR* and

TABLE 1 Conjugation factor is required for *TraR*-mediated induction of *Tra* gene expression

Test plasmid	β -Galactosidase activity from <i>tra::lacZ749</i> ^a		
	No addition	+ CF†	+ AI‡
None	<1.0	<1.0	NT
pTiC58	<1.0	<1.0	NT
pTiC58 $\Delta accR$	200.0	200.3	NT
pTiC5810-23	128.5	133.1	NT
pSa152	<1.0	<1.0	<1.0
pSVB33	<1.0	419.2	43.5
pSVB33-23	<1.0	460.7	34.5
pKP33 ΔEc	<1.0	<1.0	<1.0

Agrobacterium tumefaciens strain NT1 (ref. 16) harbouring the *tra::lacZ* reporter plasmid pJM749 was used for all constructions. Test plasmids were introduced into this strain by transformation¹⁷ or by electroporation¹⁸.

^a Expressed as Miller units¹⁹. Values less than 1.0 are insignificant. Strains were grown in AB minimal mannitol medium²⁰ at 28 °C to mid-exponential phase ($\sim 5 \times 10^8$ cells per ml). Cultures were adjusted to an optical density at 600 nm of 0.4 ($\sim 2 \times 10^8$ cells per ml) and split in half. The inducer (CF or AI) to be tested was added (25 μ l per ml) to one subculture, and the pairs of subcultures were incubated for an additional 6 h. Cells were collected by centrifugation and assayed for β -galactosidase activity as previously described⁴.

† A crude preparation of CF was prepared from *A. tumefaciens* strain NT1(pTiC58 $\Delta accR$). The strain was grown to saturation in AB mannitol medium at 28 °C. Cells were removed by centrifugation, and the culture supernatant was sterilized by filtration. 25 μ l of this preparation elicited a maximum response when added to 1.0 ml of a culture of a reporter strain harbouring pJM749 and pSVB33.

‡ A crude preparation of AI was prepared from *E. coli* strain JM109 harbouring pJE202 (Gift of E. P. Greenberg). This recombinant plasmid encodes the entire *lux* regulon of *V. fischeri*²¹. The strain was grown to saturation in LB medium at 28 °C and the culture was processed as described above for CF. NT indicates not tested.

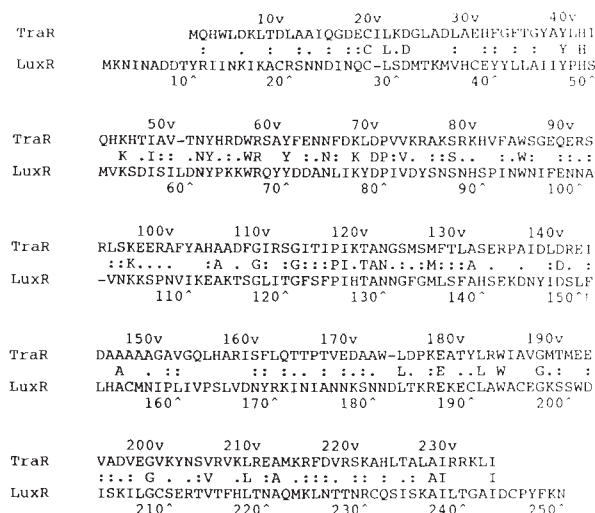


FIG. 2 Amino-acid sequence similarity between TraR and LuxR. The entire sequences of the two proteins are presented using the single-letter amino-acid code. Identities between the two sequences are shown by letter. (Colon indicates a conserved change at a given position, dot indicates a neutral change.)

METHODS. The derived amino-acid sequence of the *traR* ORF was determined from the primary DNA sequence using DNA Strider and compared with all peptide and protein sequences in the GenBank, EMBL, SWISS-PROT, and GenPept data banks using the FASTA program²⁴. Similarities were detected between TraR and the LuxR protein, and the two amino-acid sequences were aligned optimally using the DNA Star program.

TrAr are related, and the cofactors, AI and CF are chemically similar^{8,14} and physiologically crossfunctional. Recently elastase production by *Pseudomonas aeruginosa* also was shown to be regulated by a LuxR-like activator¹⁵. Although the structure of the soluble coinducer is not known, it is probably similar to AI and CF. This degree of relatedness between the systems of three dissimilar bacteria suggests that autoinduction mediated by a gene activator protein and a cognate substituted homoserine lactone coinducer constitutes an environmentally responsive regulatory mechanism that may be common among microorganisms. □

Long-term correction of rat model of Parkinson's disease by gene therapy

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THE implantation of cells genetically modified to express tyrosine hydroxylase has been proposed for the treatment of Parkinson's disease¹. Tyrosine hydroxylase converts tyrosine to L-DOPA and endogenous decarboxylase activity then converts L-DOPA to the neurotransmitter dopamine, which alleviates the symptoms of Parkinson's disease. Immortalized cells have been successfully used as intracerebral vehicles for transgene expression of tyrosine hydroxylase, but the tumorigenic potential of these cells prevents their application in humans¹⁻⁴. Intracerebral expression of this enzyme has also been achieved using primary cells like skin fibroblasts⁵⁻⁷, but the ameliorating effect on a rat model for Parkinson's disease lasted for only a few weeks. We have found that co-transplantation of cultured myoblasts and myotubes enabled reporter genes to be expressed intracerebrally at high and stable levels⁸⁻¹⁰. Here we show that the intracerebral transplantation of plasmid-transfected primary muscle cells can substantially reduce for the long-term the asymmetric rotational behaviour in the rat model.

Two days after primary rat muscle cells were lipofected with a tyrosine hydroxylase expression plasmid (pCMVTH), about 20% of myoblasts in 2-day-old cultures and ~50% of myotubes in 8-day-old cultures contained enzyme immunoreactivity (Fig. 1a). After pCMVTH-lipofected myoblasts and myotubes were co-transplanted into rats with unilateral striatal denervation, staining for tyrosine hydroxylase was seen in the intact striatum and in the muscle grafts (Fig. 1b and c). Some 40% of muscle cells were still immunoreactive for tyrosine hydroxylase at 2 months (2 animals), 4 months (2 animals) and 6 months (3 animals); muscle grafts were $\sim 0.13 \times 1.4 \times 2.2$ mm in maximum diameter at these times. Neuronal fibres positive for endogenous enzyme were observed adjacent to some of the grafts. In rats with nigrostriatal lesions, control grafts of pRSVLacZ-lipofected muscle cells were immunoreactive for β -galactosidase (data not shown) but not for tyrosine hydroxylase (Fig. 1d and e). These results agree with the very efficient transfection found for reporter genes (luciferase and β -galactosidase) in cultured muscle cells⁸ and with the high and stable expression of reporter genes after intracerebral co-transplantation of lipofected myoblasts and myotubes⁹. In addition, plasmid expression was stable for at least 19 months after intramuscular injection in mice¹¹.

After pCMVTH-lipofected muscle cells were implanted, the mean contralateral rotations induced by apomorphine decreased significantly (*t*-test, $P < 0.001$) by 75% from 15.2 times per min ($n = 26$, s.e.m. ± 0.48) to 3.8 times per min ($n = 11$, s.e.m. ± 0.8) for at least 6 months after transplantation (Fig. 2a). Although it was not clear why the number of rotations decreased with time after transplantation, no tumours were found. There was no decrease in contralateral rotation in control rats that received pRSVLacZ-lipofected muscle cell implants (Fig. 2a), which suggests that reduction of asymmetrical rotational behaviour was due to tyrosine hydroxylase expression and not to an effect of the muscle cells. In addition, contralateral rotations were reduced to the same extent in animals with or without positive neuronal fibres adjacent to the grafts. Removal of the grafts in

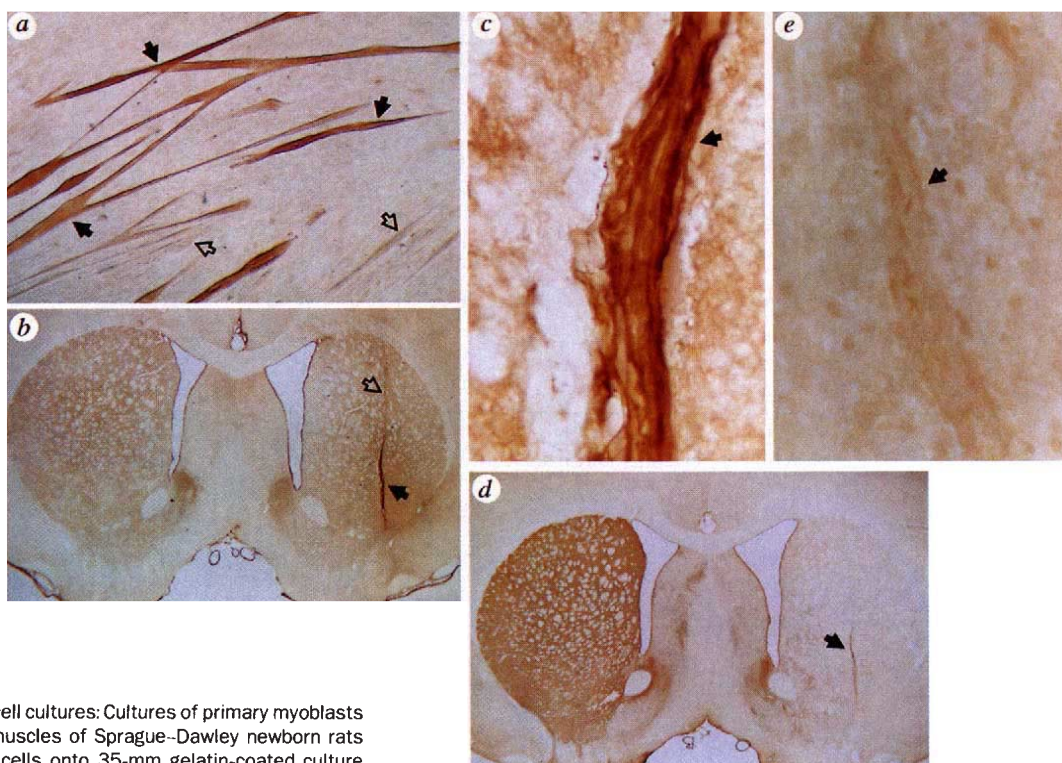
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1. Ellis, J. G. & Murphy, P. J. *Molec. gen. Genet.* **181**, 36-43 (1981).
2. Ryder, M. H., Tate, M. E. & Jones, G. P. *J. biol. Chem.* **259**, 9704-9710 (1984).
3. Ellis, J. G., Kerr, A., Petit, A. & Tempé, J. *Molec. gen. Genet.* **186**, 269-274 (1982).
4. Hayman, G. T. & Farrand, S. K. *J. Bact.* **170**, 1759-1767 (1988).
5. Beck von Bodman, S., Hayman, G. T. & Farrand, S. K. *Proc. natn. Acad. Sci. U.S.A.* **89**, 643-647 (1992).
6. Beck von Bodman, S., McCutchan, J. E. & Farrand, S. K. *J. Bact.* **171**, 5281-5289 (1989).
7. Zhang, L. & Kerr, A. *J. Bact.* **173**, 1867-1872 (1991).
8. Zhang, L., Murphy, P. J., Kerr, A. & Tate, M. E. *Nature* **362**, 446-448 (1993).
9. Sanger, G., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
10. Depicker, A. *et al. Plasmid* **3**, 193-211 (1980).
11. Engebrecht, J. & Silverman, M. *Nucleic Acids Res.* **15**, 10455-10467 (1987).
12. Neilson, K. H., Platt, T. & Hastings, J. W. *J. Bact.* **104**, 313-322 (1970).
13. Dunlap, P. V. & Greenberg, E. P. in *Microbial Cell-Cell Interactions* (ed. Dworkin, M.) 219-253 (Am. Soc. Microbiol., Washington DC, 1991).
14. Eberhard, A. *et al. Biochemistry* **20**, 2444-2449 (1981).
15. Gambello, M. J. & Iglewski, B. H. *J. Bact.* **173**, 3000-3009 (1991).
16. Watson, B., Currier, T. C., Gordon, M. P., Chilton, M.-D. & Nester, E. W. *J. Bact.* **123**, 255-264 (1975).
17. Holsters, M. *et al. Molec. gen. Genet.* **163**, 181-187 (1978).
18. Cangelosi, G. A., Best, E. A., Martinetti, G. & Nester, E. W. *Meth. Enzymol.* **204**, 384-397 (Academic, San Diego, 1991).
19. Miller, J. H. *Experiments in Molecular Genetics* 352-359 (Cold Spring Harbor Laboratory Press, New York, 1972).
20. Chilton, M.-D. *et al. Proc. natn. Acad. Sci. U.S.A.* **71**, 3672-3676 (1974).
21. Engebrecht, J., Neilson, K. & Silverman, M. *Cell* **32**, 773-781 (1983).
22. Stachel, S. E., An, G., Flores, C. & Nester, E. W. *EMBO J.* **4**, 891-898 (1985).
23. Tate, R. C. *et al. Bio/Technology* **1**, 269-275 (1983).
24. Pearson, W. R. & Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2444-2448 (1988).

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FIG. 1 Immunohistochemical staining for tyrosine hydroxylase (TH) expression in pCMVTH-lipofected myotubes in culture (a) and in brains of rats with nigrostriatal lesions 6 months after transplantation of pCMVTH-transfected (b and c) or pRSVLacZ²¹-transfected muscle cells (d and e). The behavioural data of these animals are included in Fig. 2. In a, filled arrows indicate TH-positive myotubes and open arrows indicate TH-negative myotubes. In b-e, filled arrows indicate graft. In b, open arrow shows the edge of a graft at another injection site. The section in d was overstained to verify that there was no TH expression in the denervated striatum and graft. Scale bars, 300 μ m in a, 2,500 μ m in b and d, 140 μ m in c and e.



METHODS. Preparation of muscle cell cultures: Cultures of primary myoblasts were prepared from the soleus muscles of Sprague-Dawley newborn rats as described⁸. After plating 10^6 cells onto 35-mm gelatin-coated culture plates, cells were maintained in complete medium (5% chick embryo extract (Gibco, BRL), 15% horse serum, 1% penicillin-streptomycin and 79% modified Eagle's medium) which was changed every other day. Gene transfer: Rat 1.7-kb TH cDNA²² (gift from K. O'Malley) was cloned into the eukaryotic expression vector cDNA-1 (Invitrogen) to form the pCMVTH vector which contains the cytomegalovirus promoter 5' to the TH gene and SV40 intron and poly(A) region. Plasmid DNA (15 μ g) and lipofectin (45 μ g; BRL) were first diluted separately into 0.75 ml Opti-MEM I (Gibco, BRL) and then mixed together drop by drop. The DNA-lipofectin mixture (1.5 ml) was added to primary muscle cell cultures which were washed 3 times with Opti-MEM I (without serum). Three hours after incubation, 1 ml complete medium was added which was changed the next day. Intracerebral transplantation of muscle cells: One or two days after lipofection, cultured muscle cells were collected using Cell Lifters (Fisher) without trypsinization. Myoblasts (3 d in

culture) and myotubes (8 d in culture) were mixed together at a 1:1 ratio and resuspended in basic medium (6% glucose in 0.9% normal saline) to yield $\sim 70,000$ vital cells per μ l for transplantation. Three 5- μ l graft injections (equivalent to a total of $\sim 10^6$ viable cells) were delivered by an 18-gauge metal cannula to the denervated caudate-putamen at the following coordinates²³ (in mm) relative to bregma and the dural surface, with the tooth-bar set at zero: (1) A=1.8, L=2.5, V=4.5; (2) A=0.6, L=2.0, V=4.5; (3) A=0.6, L=3.2, V=4.5. Immunohistochemical staining: The muscle cells in culture and 40- μ m brain sections were stained for TH immunoreactivity using a rabbit anti-TH antibody (Eugene Tech), a biotinylated goat anti-rabbit IgG antibody (Sigma) and avidin-conjugated HRP complex (Vector) according to the manufacturers' recommendations.

the experimental group increased contralateral rotation, which indicates that the long-term effect of the transplanted cells is due to their continued presence (Fig. 2b and c). There was no change in rotational behaviour after the pRSVLacZ-lipofected muscle grafts were removed from two control animals (data not shown). Histological analysis after completion of behavioural testing indicated that the muscle grafts had been totally removed by aspiration.

Synthesis of L-DOPA and of dopamine in the pCMVTH-lipofected cells was investigated to determine the basis of the behavioural effects. Medium from pCMVTH-lipofected myotubes (Table 1) and from myoblasts (data not shown) supplemented with DL-6-methyl-5,6,7,8-tetrahydropterine (BH4) contained substantial amounts of L-DOPA and dopamine (Table 1). Extracts from pCMVTH-lipofected cells had 20- to 30-fold less L-DOPA and dopamine than their media. Control muscle cells lipofected with pRSVLacZ did not contain any L-DOPA or dopamine (Table 1). For comparison, conditioned medium from 10^6 primary fibroblasts lipofected with pCMVTH contained 65.2 ng ml⁻¹ (± 9.7 ; $n = 4$) L-DOPA, but no dopamine; conditioned medium from primary rat adrenal medulla cultures contained 97.2 ng ml⁻¹ ($n = 2$) dopamine, but only 3.7 ng ml⁻¹ L-DOPA. The ability of muscle cells to secrete dopamine may be due to increased decarboxylase activity, decreased degradation, or increased secretion. *Xenopus* myocytes release micro-injected acetylcholine in a quantal fashion¹², so it is possible

TABLE 1 Mean L-DOPA and dopamine in conditioned media of 8-day-old muscle cell cultures containing myotubes

Plasmid lipofected	L-DOPA (ng ml ⁻¹)		Dopamine (ng ml ⁻¹)	
	-BH4	+BH4	-BH4	+BH4
pCMVTH	<0.5 ($n = 3$)	59.5 $\pm$ 35.0 ($n = 6$)	5.3 $\pm$ 4.3 ($n = 3$)	221.8 $\pm$ 25.8 ($n = 6$)
pRSVLacZ	<0.5 ($n = 4$)	<0.5 ($n = 4$)	<0.5 ($n = 4$)	<0.5 ($n = 4$)
None	<0.5 ($n = 6$)	<0.5 ($n = 6$)	<0.5 ($n = 6$)	<0.5 ($n = 6$)

Lipofected muscle cells were assayed for production and release of L-DOPA and dopamine 24 h after exposure to 500 μ M DL-6-methyl-5,6,7,8-tetrahydrobiopterin (BH4) and three days after lipofection. Conditioned media were assayed for the catecholamines using a protocol (V.G. *et al.*, manuscript in preparation) that combines and optimizes methods of sample preparation by ultrafiltration¹⁸, ion-pair HPLC separation¹⁹ and direct fluorometric detection (excitation at 280 nm and emission at 315 nm)²⁰. Conditioned media (450 μ l of 2 ml total) were collected into 50 μ l 1 M perchloric acid, dihydroxybenzylamine (100 ng in 10 μ l; DHBA, Aldrich) was added as internal standard, and the sample was filtered through an ultrafiltration membrane (cutoff at M_r 5K; Ultrafree-MC, Millipore). For HPLC, a ternary gradient solvent delivery system was used with an adsorbosphere catecholamine column (Alltech); isocratic elution was with degassed 3% isopropanol in 20 mM sodium phosphate, pH 3.0, with 50 mg l⁻¹ SDS and 50 mg l⁻¹ Na₂EDTA at 260 bar pressure and 45°C.

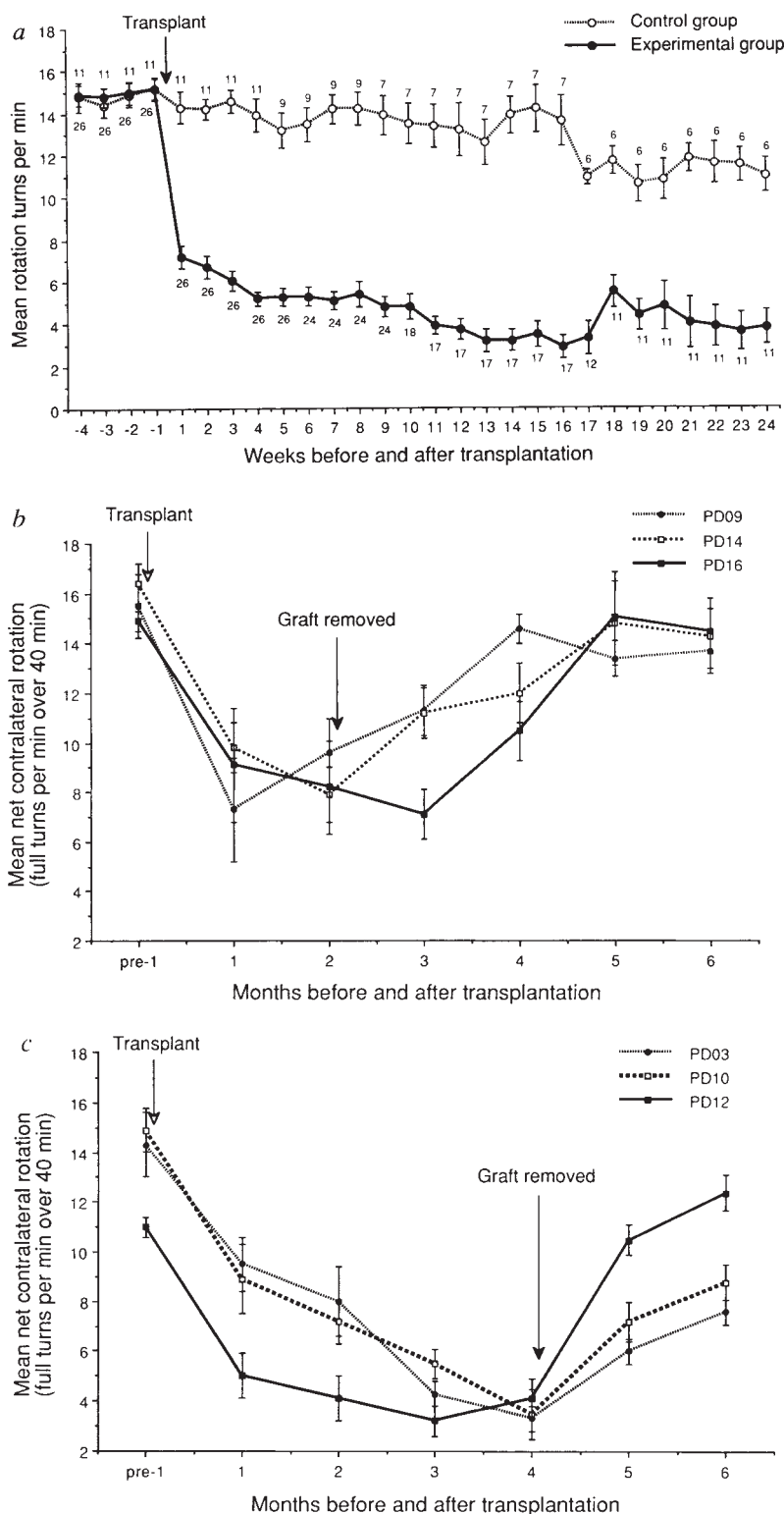


FIG. 2 Apomorphine-induced, asymmetrical, rotational behaviour in rats with nigrostriatal lesions before and after receiving implants of pCMVTH-lipofected muscle cells (a, filled circles) or pRSVLacZ (a, open circle). In some rats the implants of pCMVTH-lipofected muscle cells were removed at 9 weeks (b) or 16 weeks (c) after transplantation. In a are shown the number of rats, the mean values and standard errors; in b and c, the rotation behaviour of individual rats was assessed every week and the mean rotations and standard errors for each 4-week period are shown. PD models are identified by number (see Table 2).

METHODS. The left ascending meso-striatal dopaminergic fibre bundle of adult female Sprague-Dawley rats (175–200 g) was stereotactically injected with 6-hydroxydopamine (Sigma) to create PD models²⁴. After a 7-d recovery period and once a week thereafter, motor asymmetry was assessed by measuring rotational behaviour with an automated rotometer for 40 min after subcutaneous injection of 0.05 mg kg⁻¹ of apomorphine hydrochloride dissolved in 0.2 mg ml⁻¹ ascorbate-saline solution. Only rats that showed more than seven turns per min for one month were selected as PD models for transplantation. The lipofected muscle cells were transplanted into the striatum as described for Fig. 1. In some rats (b and c), grafts were stereotactically removed by aspiration with a 9-gauge cannula. The brain cavity was filled with gelfoam.

that rat muscle cells might also regulate neurotransmitter secretion.

We excised the striatal areas, including the grafts, and assayed for L-DOPA and dopamine at varying times after transplantation in experimental and control animals (Table 2). Rat models for Parkinson's disease (PD) receiving pCMVTH-transfected muscle grafts had striatal dopamine levels similar to those in control animals without nigrostriatal lesions for at least 4 months after transplantation. The striatal areas of rat PD models without grafts, with untransfected muscle grafts, or with pRSVLacZ-transfected muscle grafts, contained very little or no dopamine.

Small amounts of L-DOPA were detected in some of the control animals without nigrostriatal lesions and in some of the experimental animals that received pCMVTH-transfected muscle grafts (Table 2). Striatal L-DOPA and dopamine levels are consistent with *in vitro* concentrations and rotational behaviour and indicate that transplantation of pCMVTH-transfected muscle cells reduces asymmetrical rotation in the lesioned animals as a result of L-DOPA and dopamine production. Transplanted cells presumably take up sufficient biotin for tyrosine hydroxylase activity from either intracerebral biotin^{13,14} or from blood biotin through a disrupted blood-brain barrier¹⁵.

TABLE 2 Striatal L-DOPA and dopamine in host brains and grafts

Experimental condition	Rat	Months post-transplant	L-DOPA (ng per mg wet tissue)	Dopamine (ng per mg wet tissue)	Reduction in rotation* (%)
Control: Substantia nigra injected with normal saline, no transplantation	N1	—	<0.1	4.4	—
	N2	—	0.7	7.9	—
	N3	—	0.3	8.0	—
	N6	—	0.7	13.2	—
Control: PD model, no graft	PD18	—	<0.1	<0.1	0
	PD19	—	<0.1	<0.1	0
Control: PD model, nontransfected muscle graft	PD35	2	<0.1	0.6	0
	PD36	2	<0.1	<0.1	0
	PD39	4	<0.1	<0.1	0
	PD40	4	<0.1	<0.1	0
	PD33	1	<0.1	0.5	0
Control: PD model, pRSVLacZ-transfected muscle graft	PD34	1	<0.1	0.8	8
	PD15	2	<0.1	<0.1	0
	PD23	1	1.1	3.4	60
Experimental: PD model, pCMVTH-transfected muscle graft	PD25	1	<0.1	2.6	43
	PD04	2	0.3	4.3	68
	PD08	2	<0.1	2.9	47
	PD03†	4	0.2	4.1	59
	PD10†	4	<0.1	6.1	74
	PD12†	4	<0.1	4.1	49
	PD17	4	<0.1	5.2	69

The behaviour of animals with pRSVLacZ- or pCMVTH-transfected muscle grafts is included in Fig. 2. Excised striatum was ground under frozen liquid N₂, shaken in a tared microfuge tube containing 250 µl 0.1M HClO₄ for 5 min and centrifuged at 13,000 r.p.m. for 15 min at 4 °C. The supernatant was ultrafiltered and analysed for catecholamine content as in Table 1.

* Per cent reduction in the mean contralateral rotations per min (averaged over the 40-min testing period) after transplantation is given by [(mean rotation before transplantation – mean rotation after transplantation and just before death)/mean rotation before transplantation] × 100.

† Rats whose grafts were removed without killing and whose rotational behaviour is detailed in Fig. 2c.

This muscle cell/gene transfer system allows long-term expression of our plasmids and correction of a rat PD model, in contrast to the unstable expression often observed with retrovirally transduced genes^{5-7,16}. Plasmid DNA is potentially safer than a viral vector. Muscle cells that can secrete dopamine as well as L-DOPA could offer a more effective treatment for human Parkinson's disease. Patients have low decarboxylase activity in their striatum and if this decarboxylase activity decreases further, L-DOPA may lose its efficacy over time¹⁷. In summary, the consistent ability of this muscle cell/gene transfer system to promote high levels of intracerebral transgene expression and substantially to reduce asymmetric rotational behaviour in the rat PD model makes it an attractive candidate for clinical application. □

Repair of demyelinated lesions by transplantation of purified O-2A progenitor cells

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THE transplantation of well defined populations of precursor cells offers a means of repairing damaged tissue and of delivering therapeutic compounds to sites of injury or degeneration. For example, a functional immune system can be reconstituted by transplantation of purified haematopoietic stem cells¹, and transplanted skeletal myoblasts and keratinocytes can participate in the formation of normal tissue in host animals²⁻⁴. Cell transplantation in the central nervous system (CNS) has been proposed as a means of correcting neuronal dysfunction in diseases associated with neuronal loss⁵⁻⁷; it might also rectify glial cell dysfunction, with transplanted oligodendrocyte precursor cells eventually allowing repair of demyelinating damage in the CNS. Here we use co-operating growth factors to expand purified populations of oligodendrocyte type-2 astrocyte (O-2A) progenitor cells for several weeks *in vitro*. When injected into demyelinating lesions

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- Wolff, J. A. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **86**, 9011-9014 (1989).
- Horellou, P. *et al.* *Neuron* **5**, 393-402 (1990).
- Stromberg, I. *et al.* *J. Neurosci. Res.* **25**, 405-411 (1990).
- Gage, F. H. *et al.* *Neuroscience* **23**, 795-807 (1987).
- Gage, F. H. & Fisher, L. J. *et al.* *Neuron* **6**, 1-12 (1991).
- Fisher, L. J. *et al.* *Neuron* **6**, 371-380 (1991).
- Chen, L. S. *et al.* *J. cell. Biochem.* **45**, 252-257 (1991).
- Jiao, S. S., Schultz, E. & Wolff, J. A. *Brain Res.* **575**, 143-147 (1992).
- Jiao, S. S. *et al.* *Cell Transpl.* (in the press).
- Jiao, S. S. & Wolff, J. A. *Neurosci. Lett.* **137**, 207-210 (1992).
- Wolff *et al.* *Hum. Molec. Genet.* **1**, 363-369 (1992).
- Dan, Y. & Poo, M. M. *Nature* **359**, 733-736 (1992).
- Birkmayer, G. J. D. & Birkmayer, W. *Acta neurol Scand.* **126**, 183-187 (1989).
- Kapatos, G., Katoh, S. & Kaufman, S. J. *Neurochem.* **39**, 1152-1162 (1982).
- Wakai, S., Meiselman, S. E. & Brightman, M. W. *Brain Res.* **386**, 209-222 (1986).
- Palmer, T. D., Rosman, G. Y., Osborne, W. R. A. & Miller, A. D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1330-1334 (1991).
- Melamed, E. in *Handbook of Parkinson's Disease* (ed. Koller, W. C.) 355-370 (Dekker, New York, 1987).
- Kamahori, M., Taki, M. & Watanabe, Y. J. *J. Chromat.* **567**, 351-358 (1991).
- Crombeen, J. P., Kraak, J. C. & Poppe, H. J. *J. Chromat.* **167**, 219-230 (1978).
- Svendsen, H. & Greibrokk, T. J. *J. Chromat.* **213**, 429-437 (1981).
- Norton, P. A. & Coffin, J. M. *Molec. cell. Biol.* **5**, 281-290 (1985).
- Brown, E. R., Coker, G. T. T. & O'Malley, K. L. *Biochemistry* **26**, 5208-5212 (1987).
- Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates* (Academic, New York, 1982).
- Perlow, M. J. *et al.* *Science* **204**, 643-647 (1979).

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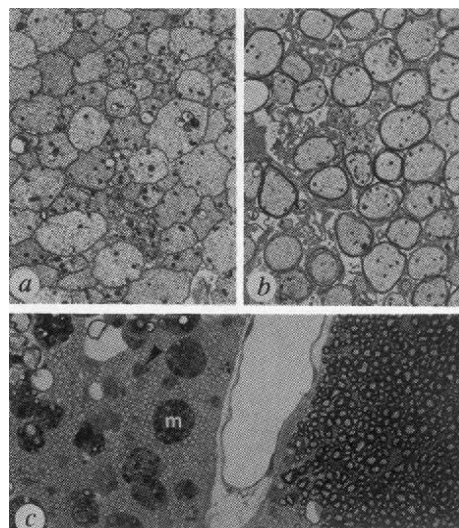
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in spinal cords of adult rats, created in such a way as to preclude host-mediated remyelination, these expanded populations are capable of producing extensive remyelination. In addition, transplantation of O-2A progenitor cells genetically modified to express the bacterial β -galactosidase gene gives rise to β -galactosidase-

positive oligodendrocytes which remyelinate demyelinated axons within the lesion. These results offer a viable strategy for the manipulation of neural precursor cells which is compatible with attempts to repair damaged CNS tissue by precursor transplantation.

FIG. 1 The environment into which O-2A progenitor cells are transplanted. It consists of a sharply defined lesion in which demyelinated axons are packed tightly together in an area devoid of glial cells, created by injecting ethidium bromide into X-irradiated spinal cord white matter (Magnification, $\times 2,500$). *b*, Three weeks after transplantation of O-2A progenitor cells, about 75% of the axons in the field shown are surrounded by a thin compact sheath of central myelin; the remaining axons are engulfed by glial processes (magnification, $\times 1,500$). From 3 to 5 sections, spaced at 1-mm intervals through the lesion, were examined from each animal. *c*, Remyelination can be seen in the area to the left of the blood vessel in the centre of this figure. The area contains transplanted cells and axons remyelinated with myelin as in *b*. The darkly stained cells (m) are macrophages containing myelin debris generated during demyelination. Remyelination extends to the edges of the lesion where it is continuous with the normal white matter to the right of the blood vessel (toluidine blue-stained resin section; magnification, $\times 150$).

METHODS. Optic nerve cultures were prepared from postnatal-day-7 (P7) Wistar rat pups as described^{8,12} and cultured in a supplemented form of Dulbecco's Modified Eagle medium (DMEM-BS; refs 8 and 12) with the addition of 10 ng ml^{-1} bFGF and 10 ng ml^{-1} of the AA homodimer of PDGF. Cultures were grown for four weeks, and were passaged after one and three weeks, yielding cultures containing $\geq 95\%$ O-2A progenitor cells. Adult rats from the same inbred colony that provided the P7 pups for the O-2A progenitor cultures were used in all transplantations. Three days before induction of demyelination, animals were X-irradiated¹⁰. Demyelination was produced in the dorsal columns of the spinal cord at the level of the first lumbar vertebra by the injection of $1 \mu\text{l}$ of 0.1% ethidium bromide¹⁰, and



$1 \mu\text{l}$ of O-2A progenitor cell suspension was injected into lesions three days later. Animals were killed 21 days after transplantation and $1\text{-}\mu\text{m}$ sections were cut from the graft region and stained with toluidine blue. Selected samples were trimmed and thin-sectioned for ultrastructural study.

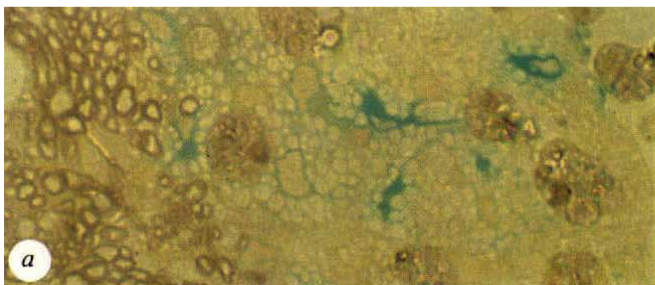
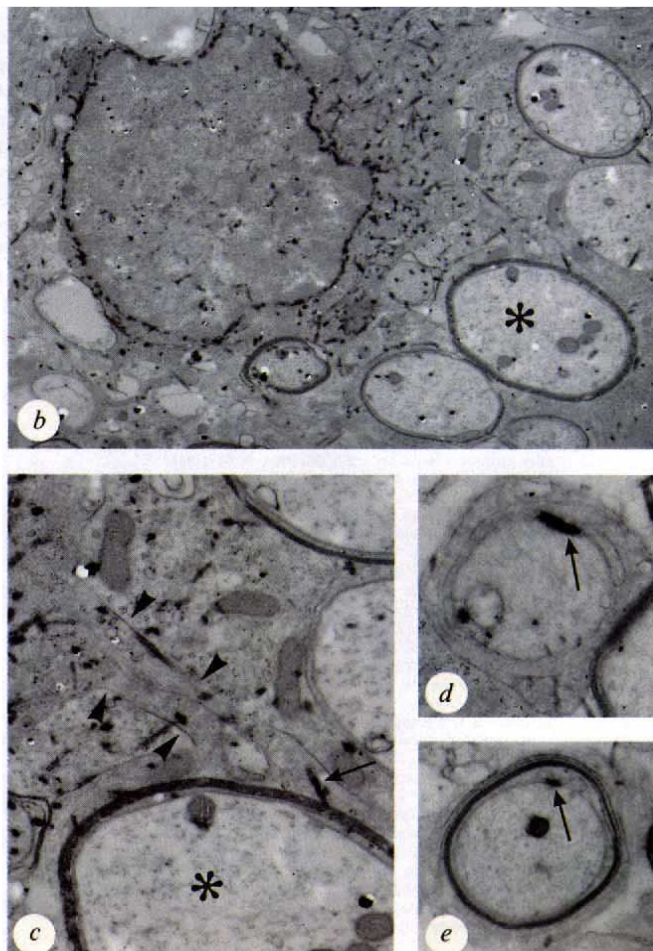


FIG. 2 *a*, Transplanted BAG-infected O-2A progenitor cells carrying the *lacZ* gene could be detected in the lesion by treating the tissue with the substrate X-gal¹⁵. Normally myelinated axons in unlesioned white matter can be seen on the left of the picture (unstained resin section; $\times 350$). *b*, Labelled cells were identified in the electron microscope by the presence of rod-shaped electron-dense structures in their cytoplasm²⁶. This labelled cell has the morphological features of an oligodendrocyte and is associated with axons that have been remyelinated by a thin sheath of central myelin ($\times 8,360$). *c*, The labelled cell in *b* is connected to the remyelinated axon (asterisk, also in *b*) by the process indicated by the arrowheads. The outer process of the oligodendrocyte contains a crystal of β -galactosidase reaction product (arrow; $\times 22,300$). *d*, After transplantation of BAG-expressing O-2A progenitor cell lines cultured for 4 months, β -galactosidase reaction product can be seen (arrow) within the process of an oligodendrocyte that is wrapped around a demyelinated axon in the early stages of remyelination ($\times 8,360$). *e*, Remyelinated axon in which a single crystal of β -galactosidase reaction product (arrow) can be seen within the inner tongue of a remyelinating oligodendrocyte ($\times 8,360$).

METHODS. O-2A progenitor cells were purified by antibody-mediated cell capture (panning)^{16,17}. Panned O-2A progenitor cell cultures were infected with the BAG retrovirus¹⁵ 1, 3 and 6 days after panning. O-2A progenitor cell lines containing the BAG DNA construct were generated by lipofectin-mediated transfection of optic nerve cultures^{24,25}. After 2 weeks of continuous growth, cells were selected with G418 until distinct colonies were visible. Colonies were then ring-cloned and expanded in culture. Twenty-one days after transplantation, animals were killed and processed as for Fig. 1. For demonstration of β -galactosidase activity, lesion-containing tissue was incubated in X-gal solution^{15,26}.



To investigate the possibility that populations of purified oligodendrocyte precursors could be used to achieve remyelination of demyelinating CNS lesions, we transplanted a variety of preparations of purified oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells⁸ into demyelinating lesions in the spinal cords of adult rats. These lesions were generated by injection of 0.1% ethidium bromide into spinal cord white matter that had been exposed to 40 Grays of X-irradiation three days previously. This lesion model consists of demyelinated axons residing in a glia-free environment which cannot be remyelinated by cells derived from the host animal⁹⁻¹¹.

In initial experiments, O-2A progenitor cells derived from optic nerves of 7-day-old rats were grown in culture in the presence of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), a condition which causes O-2A progenitor cells to proliferate continuously in the absence of differentiation into oligodendrocytes¹². Cells were passaged after one week in culture, and again after a further two weeks. This procedure routinely yielded cultures containing >95% O-2A progenitor cells, with contaminating cells consisting primarily of astrocytes and macrophages; the method of culture used in these experiments generated cultures that contained $\leq 1\%$ oligodendrocytes (as defined by immunolabelling with monoclonal anti-galactocerebroside antibody^{13,14}). A suspension of 6×10^4 cells in $1 \mu\text{l}$ was injected into spinal lesions of ten syngeneic adult rats, with a series of non-transplanted lesioned animals and animals injected with medium alone serving as controls. Three weeks after transplantation, the lesioned area of the spinal cord was examined by light and electron microscopy (Fig. 1a-c).

Eight out of the ten lesioned animals receiving injections of O-2A progenitor cells showed clear evidence of repair, with a mean of $60 \pm 7.3\%$ (s.e.m.) of the demyelinated axons identified in representative sections (cut at 1-mm intervals throughout the lesion) being remyelinated with central myelin (Fig. 1b, c). In 4 of the 8 animals, $\geq 90\%$ of the axons were remyelinated in several sections from each animal.

We confirmed that the remyelinating oligodendrocytes in our experimental lesions were unequivocally derived from the transplanted cells by using infection with the BAG retrovirus¹⁵ to introduce the bacterial β -galactosidase gene into O-2A progenitor cells whose purity had been enhanced to >99.5% by antibody-mediated cell capture (panning^{16,17}). Panned cultures of O-2A progenitor cells, continually grown in the presence of PDGF and bFGF, were infected with the BAG retrovirus¹⁵ on alternate days for 6 days to achieve a high level of β -galactosidase expression in the absence of antibiotic selection. (At the time of injection, 18% of the O-2A progenitors expressed β -galactosidase protein, as determined by immunolabelling analysis¹⁸.) Three lesioned animals received injections of uninfected cells, and three lesioned animals received retrovirally infected cell transplants. Injected populations consisted of >99.5% O-2A progenitors, with a very small proportion of

contaminating oligodendrocytes, as determined by immunolabelling of cells prepared from the same cultures but grown in parallel on coverslips. Both groups of animals showed central remyelination that was similar to, but less extensive than, that seen in the first experiment ($46 \pm 4.7\%$). In two out of three of the animals receiving genetically modified cells, 60–80% of the axons were remyelinated in several sections from each animal. In the group that received infected cells, the percentage of myelin-producing cells that were labelled blue was roughly the same as the percentage of β -galactosidase-expressing cells present in the transplant suspension. No difference was detected in the myelin sheaths associated with blue labelled cells compared with those produced by unlabelled cells (Fig. 2a-c). It is of interest that we did not see β -galactosidase-expressing cells with the morphological characteristics of astrocytes in any of our transplantation experiments.

We next examined the capacity of O-2A progenitor cell lines to remyelinate X-irradiated ethidium bromide lesions. These cell lines were obtained by growth of O-2A progenitor cells that had been lipofected with the BAG DNA construct, and grown in culture for four months after selection for neomycin resistance (transduced by the BAG construct¹⁵) and cloning of antibiotic-resistant colonies. At the end of this time, cloned cultures were still able to differentiate readily into oligodendrocytes *in vitro* (data not shown). Transplantation of these cells resulted in small areas of remyelination in which it was possible to identify oligodendrocytes distinctively labelled with the β -galactosidase reaction product (Fig. 2d, e). However, in contrast to the short-term cultures, many of the transplanted cells did not differentiate into myelinating cells within our 3-week period, and their continued proliferation resulted in distortion of the surrounding normal host tissue.

Our results extend previous demonstrations^{9-11,19-23,27} of remyelination by transplantation of glial cells in several ways: (1) the use of co-operating growth factors to generate large numbers of O-2A progenitors shows that it is possible to create populations of these cells suitable for transplantation and genetic manipulation without recourse to the production of immortalized cell lines; (2) the use of highly purified populations of O-2A progenitors shows that transplantation of a heterogeneous population of glial cell types is not necessary to obtain extensive remyelination in the CNS; (3) the genetic modification of O-2A progenitors to express β -galactosidase shows unequivocally that myelinating oligodendrocytes are generated from our transplanted O-2A progenitor cells and also that these cells can be engineered to express a foreign protein while still retaining function. In common with other transplantation experiments^{2-4,6,7}, however, we do not yet know whether the myelin repair seen in our lesion model is associated with functional recovery from conduction block in the remyelinated axons. It is our view, however, that these experiments represent an important step in the development of repair strategies for demyelinated lesions of the CNS. □

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- Spangude, G. J., Heimfeld, S. & Weissman, I. L. *Science* **241**, 58–62 (1988).
- Gussoni, E. *et al.* *Nature* **356**, 435–438 (1992).
- Dhawan, J. *et al.* *Science* **254**, 1509–1512 (1991).
- Morgan, J. R., Barrandon, Y., Green, H. & Mulligan, R. C. *Science* **237**, 1476–1479 (1987).
- Lindvall, D. *et al.* *Science* **247**, 574–577 (1990).
- Renfranz, P. J., Cunningham, M. J. & McKay, R. D. G. *Cell* **66**, 713–729 (1991).
- Snyder, E. Y. *et al.* *Cell* **68**, 33–51 (1992).
- Raff, M. C., Miller, R. H. & Noble, M. *Nature* **303**, 390–396 (1983).
- Blakemore, W. F. & Crang, A. J. *Dev. Neurosci.* **10**, 1–11 (1988).
- Blakemore, W. F. & Crang, A. J. *J. Neurocytol.* **18**, 519–528 (1989).
- Crang, A. J. *et al.* *J. Neuroimmun.* **40**, 243–254 (1992).
- Bögler, O., Wren, D. R., Barnett, S. C., Land, H. & Noble, M. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6368–6372 (1990).
- Raff, M. C. *et al.* *Nature* **274**, 813–816 (1978).
- Ranscht, B., Clapshaw, P. A., Price, J., Noble, M. & Siefert, W. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2709–2713 (1982).
- Price, J., Turner, D. & Cepko, C. L. *Proc. natn. Acad. Sci. U.S.A.* **84**, 156–160 (1987).
- Barres, B. A. *et al.* *Cell* **70**, 31–46 (1992).

- Mayer, M., Bögler, O. & Noble, M. (in the press).
- Williams, B. P., Read, J. & Price, J. *Neuron* **7**, 685–693 (1991).
- Duncan, I. D. *et al.* *J. Neurocytol.* **17**, 351–360 (1988).
- Franklin, R. J. M., Crang, A. J. & Blakemore, W. F. *J. Neurocytol.* **20**, 420–430 (1991).
- Gransmuller, A., Clerin, E., Kruger, F., Gumpel, M. & Lachapelle, F. *Glia* **4**, 580–589 (1991).
- Gumpel, M., Baumann, N., Raoul, M. & Jacque, C. *Neurosci. Lett.* **37**, 307–311 (1983).
- Rosenbluth, J., Hasegawa, M., Shirasaki, N., Rosen, C. L. & Lui, Z. *J. Neurocytol.* **19**, 718–730 (1990).
- Gorman, C. F., Moffat, L. F. & Howard, B. H. *Molec. cell. Biol.* **2**, 1044–1054 (1982).
- Felgner, P. L. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **84**, 7413–7417 (1987).
- Franklin, R. J. M. & Barnett, S. C. *Acta Neuropath.* **81**, 686–687 (1991).
- Warrington, A. E., Barbaresi, E. & Pfeiffer, S. E. *J. Neurosci. Res.* **34**, 1–13 (1993).

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Regulation of apical transport in epithelial cells by a G_s class of heterotrimeric G protein

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THE role of heterotrimeric GTP-binding proteins in signal transduction is well established¹. They might also be involved in vesicular transport²⁻⁶. Here we show that in the epithelial cell line Madin-Darby Canine Kidney, transport of influenza haemagglutinin protein to the apical surface is stimulated and that of vesicular stomatitis virus glycoprotein to the basolateral surface is retarded by $AlF_{(3-5)}$ treatment. Treatment of cells with the reagents known to influence the G_i class of G proteins affected only the basolateral pathway whereas reagents acting on the G_s class of G proteins specifically affected the apical pathway. In permeabilized cells, antibodies raised against the N-terminal domain of the α -subunit of G_s inhibited the transport of haemagglutinin from the *trans*-Golgi network to apical surface but not between the endoplasmic reticulum and Golgi complex. These observations demonstrate involvement of a G_s class of heterotrimeric G proteins, besides that of the G_i , in vesicular transport². Moreover, the apical and the basolateral pathways in epithelial cells seem to be regulated by G_s and G_i proteins, respectively, in the *trans*-Golgi network.

In polarized epithelial cell lines such as Madin-Darby Canine Kidney (MDCK) cells, the haemagglutinin protein (HA) of influenza virus is targeted to the apical domain whereas vesicular stomatitis virus glycoprotein (VSV-glycoprotein) is transported to the basolateral domain. Thus these two proteins have been widely used as markers for the apical and basolateral pathways, respectively⁷. To determine the role of heterotrimeric GTP-binding proteins (G protein) in the polarized delivery of proteins in these two pathways, we studied the effects of several G-protein-specific reagents. The effects of membrane-permeable reagents (such as $AlF_{(3-5)}$ and toxins) was studied on intact cells while that of membrane-impermeable reagents (such as peptides and antibodies) was studied on permeabilized cells. MDCK cells infected with influenza or VSV were treated with $AlF_{(3-5)}$, a general 'activator' of the heterotrimeric G proteins but not the small GTP-binding proteins⁸. The $AlF_{(3-5)}$ treatment resulted in stimulation of HA transport to the apical surface (Fig. 1a) and inhibition of VSV-glycoprotein to the basolateral surface (Fig. 1b). Replacement of fluoride with chloride did not show any of the $AlF_{(3-5)}$ effects (data not shown), showing the specificity of the $AlF_{(3-5)}$ effect and indicating a possible involvement of heterotrimeric G proteins.

Another hallmark of trimeric G proteins is their sensitivity to ADP-ribosylation by certain toxins^{1,9}. Treatment of intact cells with pertussis toxin (PTX), for example, results in ADP-ribosylation of the α -subunit of the inhibitory class of G protein (G_i). The α -subunit of the stimulatory class of G protein (G_s), however, is not a substrate for the action of this toxin¹⁰. Conversely, cholera toxin (CTX) ADP-ribosylates the G_s and not the G_i . Thus, treatment with these toxins has been used to distinguish the involvement of G_s from that of G_i in a given cellular function. Treatment of MDCK cells with PTX resulted in increased basolateral secretion of proteins whereas the CTX treatment caused stimulation of the apical secretion (Fig. 2a; left panel). Secretion from the opposite sides was, in contrast, not significantly affected by either of these toxins. Consequently, treatment of cells with increasing amounts of CTX resulted in increased ratios of apical to basolateral secretion whereas the

PTX treatment resulted in decreased ratios of apical to basolateral secretion (Fig. 2a; right panel).

Mastoparan (MAS), a tetradecapeptide found in bee venom, is shown to activate the α -subunit of G_i in a way analogous to an activated receptor¹¹ but is a poor stimulator of G_s (ref. 12). We then studied the effect of MAS on the polarized transport in streptolysin O (SLO)-permeabilized MDCK cells^{13,14}. When 10 μ M MAS was added to such cells from the permeabilized surface, VSV-glycoprotein transport to the basolateral surface was significantly inhibited while delivery of HA to the apical domain remained unaffected (Fig. 2b). Short amphipathic peptides such as MAS may affect a transport process in a nonspecific manner due to its side effects on general membrane structure. But the observation that the apical transport remains unaffected by MAS gives credence to our interpretation that the basolateral inhibition is a specific action of MAS. The effects of $AlF_{(3-5)}$, PTX and MAS on the basolateral trafficking show that a G_i class of the heterotrimeric G protein inhibits transport to the basolateral surface, a finding that is consistent with and extends an earlier observation². But the novel finding that $AlF_{(3-5)}$ and CTX stimulate the apical transport of HA and secretory proteins whereas the PTX or MAS treatment of cells has no effect on the apical delivery of proteins suggests that the putative heterotrimeric G protein involved in the apical trafficking belongs to the G_s class.

To provide direct evidence for the involvement of a G_s protein in the apical transport, we modified the *in vitro* system to make transport of HA protein from TGN to the apical surface dependent on exogenous addition of cytosol. Under such conditions, transport of HA protein from the *trans*-Golgi network (TGN)

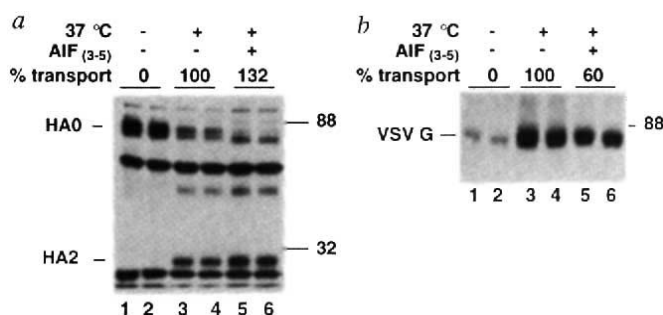


FIG. 1 $AlF_{(3-5)}$ treatment stimulates the HA transport to the apical surface (a) and inhibits the VSV-glycoprotein transport to the basolateral surface (b). METHODS. MDCKII cells were grown on 24-mm Costar filters (from H. Lane, Costar Corp.) for 3 days before being infected with influenza or VSV. The cells were pulse-labelled with ³⁵S-methionine for 6 min and the viral glycoproteins were blocked for 60 min at 20 °C in the TGN as described¹⁴. The filters were then placed on a 0.1-ml drop of chase medium containing 50 μ M ammonium aluminium sulphate and 10 mM potassium fluoride (0.5 ml of the same medium was applied to the apical side) for 10 min at 4 °C followed by 45 min at 37 °C in the continued presence of the reagents. Arrival of HA at the apical surface was measured by its sensitivity to cleavage by trypsin²² and that of VSV-glycoprotein at the basolateral surface by surface biotinylation²³ followed by binding to streptavidin-agarose resin. The trypsin-treated or streptavidin-agarose-bound material was analysed by SDS-PAGE, autoradiographed and the band intensities were calculated using PhosphorImager (Molecular Dynamics). Duplicate filters were left at 4 °C (lanes 1, 2) or incubated at 37 °C for 45 min in the absence (lanes 3, 4) or presence of $AlF_{(3-5)}$ (lanes 5, 6). The amount of HA transported to the apical surface was calculated as described before²² (% transport = $2 \times HA2/HA0 + 2HA2 \times 100$). The amount of VSV-glycoprotein transported to the basolateral surface was calculated as the amount of VSV-glycoprotein bound to streptavidin-agarose resin. The values are expressed with transport in control cells being 100% and that left at 4 °C being 0%. This and all other experiments were done in duplicate at least twice and the quantifications represent the means of one representative experiment. The value for stimulation of HA transport from five independent experiments done in duplicates is 129 ± 5.3 . The stimulation of HA transport to the apical surface was observed at all time points up to 45 min chase.

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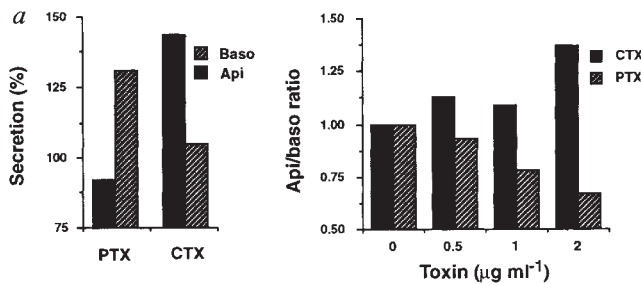
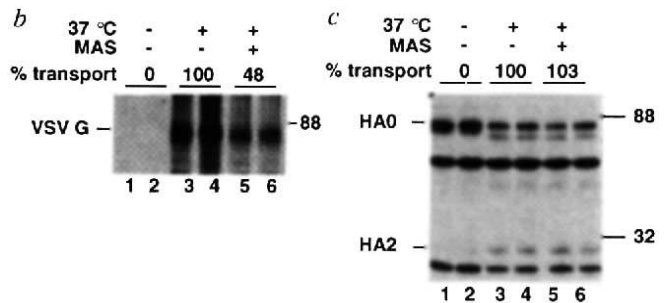


FIG. 2 *a*, Pertussis- and cholera toxin-treated MDCK cells show enhanced secretion of proteins on the basolateral and apical side, respectively. *b*, *c*, MAS inhibits the basolateral transport of VSF-glycoprotein (*b*) but not the apical transport of HA (*c*) in permeabilized cells.

METHODS. Left panel: Filter-grown cells were treated with $2 \mu\text{g ml}^{-1}$ PTX or CTX for 3 h at 37°C . The cells in duplicate filters were then pulsed with ^{35}S -methionine for 15 min and chased for 60 min at 37°C . The apical and basolateral medium was collected and triplicate aliquots were TCA precipitated and counted. The values are normalized to secretion in untreated cells as being 100%. The values are mean of the duplicate samples. Closed bars, apical secretion; hatched bars, basolateral secretion. Right panel: The experiment was done exactly as described above except the cells were treated with 0.5, 1.0 or $2 \mu\text{g ml}^{-1}$ toxin and, after the pulse-labelling, the cells were chased for 30 min. The results are shown as a ratio of apical to basolateral (api/baso) secretion and expressed as that in control cells being 1. In control cells the secretory activity (TCA precipitable counts in the medium) in apical



direction (5,667 c.p.m.) was the same as in the basolateral direction (5,706 c.p.m.) after 30 min of chase. The stimulation of basolateral and apical secretion was observed at all time points up to 120 min of chase. *b*, *c*, Filter-grown MDCK cells were infected with the viruses, pulse labelled and chased as in Fig. 1. The VSV-infected cells in duplicate filters were treated with SLO at 4°C for 15 min on the apical side while the influenza-infected cells were treated on the basolateral side. After washing the unbound SLO, the cells were incubated for 45 min in the presence of an ATP-regenerating system¹⁴ either at 4°C (lanes 1, 2) or at 37°C in the absence (lanes 3, 4) or presence of $10 \mu\text{M}$ MAS (lanes 5, 6). The ATP-regenerating system and MAS in transport medium¹⁴ were added to the permeabilized side (0.5 ml on the apical and 0.1 ml on the basolateral side) while the intact surface received only transport medium. Under such conditions the opposite surface and the junctional contacts remain intact¹⁴. The transport was measured and the values were expressed as in Fig. 1.

to the apical surface was dependent on temperature, addition of cytosol, and supply of energy (Fig. 3a). Figure 3b shows that the appearance of HA on the apical surface was inhibited when the cytosol was preincubated with the antibodies raised against the sequence KQLQKDKQVYRA (single-letter amino-acid code) near the N terminus of $\text{G}\alpha_s$ (ref. 15; referred to here as N-terminal antibodies). But the antibodies raised against the sequence ATPEPGEDPRVTRA near the C terminus of $\text{G}\alpha_s$ did not affect the transport of HA to the surface (both antibodies were from H. Bourne).

The sorting of apical and basolateral proteins does not take place during their passage from the endoplasmic reticulum (ER) to Golgi¹⁶ but takes place only on reaching the TGN, where they are packaged in two distinct types of vesicles¹⁷. We wanted to study whether an early step in the vesicular transport where

HA protein is not sorted from the basolateral proteins will also be similarly affected by the N-terminal antibodies. The conditions of the *in vitro* assay were further modified to follow transport of HA from the ER to *cis*/medial Golgi compartments by observing acquisition of Endo H resistance by HA¹⁸. Figure 4a shows that although HA acquired Endo H resistance in a cytosol- and energy-dependent manner, inclusion of the N-terminal antibodies did not inhibit this process to any significant extent. Consistent with this finding is the observation that AIF₍₃₋₅₎ treatment of the intact cells did not result in stimulated transport of HA from the ER to Golgi (Fig. 4b). The observation that the N-terminal antibodies did not inhibit this step in the transport of HA also rules out any possibility of a nonspecific effect of the antibody on vesicular traffic in general. The inhibition of TGN to apical surface transport by the anti-N-terminal

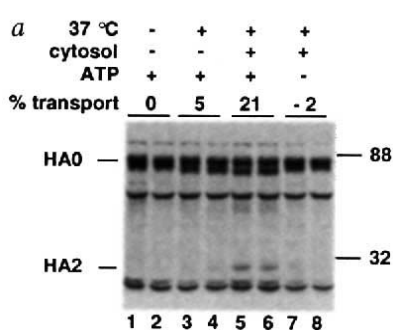
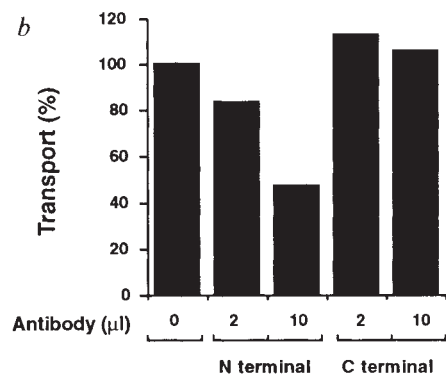


FIG. 3 *In vitro* transport of HA from the TGN to apical surface is inhibited by antibodies raised against an N-terminal peptide of $\text{G}\alpha_s$.

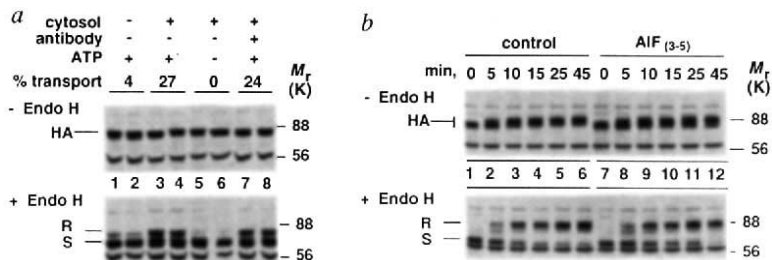
METHODS. *a*, The *in vitro* transport of HA was done on basolaterally permeabilized and cytosol-depleted MDCK cells with the following modifications to the procedure described elsewhere¹⁴. After depleting the cytosol from the cells, the transport reaction was done by placing duplicate filters on a 100- μl drop of transport medium containing the ATP-regenerating system either at 4°C (lanes 1, 2) or at 37°C in the absence (lanes 3, 4) or presence of $100 \mu\text{l}$ of HeLa cytosol instead of MDCK cytosol¹⁴ (prepared in transport medium as an 8 mg protein ml^{-1} high-speed supernatant from HeLa cells grown in suspension; lanes 5–8) in the absence (lanes 5, 6) or presence of



the ATP-depleting system (lanes 7, 8; instead of the ATP-regenerating system, these cells received hexokinase and glucose¹⁴). The apical surface was covered with 0.5 ml transport medium throughout the transport reaction. The extent of transport was calculated as described before²². *b*, The *in vitro* transport of HA was done as above in the absence or presence of exogenously added $100 \mu\text{l}$ cytosol (4 mg protein ml^{-1}) that was pre-incubated at 4°C for 60 min without or with 2 or $10 \mu\text{l}$ anti-C-terminal or 2 or $10 \mu\text{l}$ anti-N-terminal antibodies¹⁵. The values are expressed as cytosol-dependent transport being 100% (transport in the presence of added cytosol minus transport in the absence of added cytosol) and are mean of the duplicates.

FIG. 4 *In vitro* transport of HA from ER to the Golgi is unaffected by the presence of the anti-N-terminal antibodies.

METHODS. *a*, Filter-grown MDCK cells were infected with influenza virus and pulse-labelled with ^{35}S -methionine for 7 min. The cells were immediately permeabilized with SLO from the basolateral side. The transport reaction was done on duplicate filters exactly as in Fig. 3a in the presence of the ATP-regenerating system at 37 °C in the absence (lanes 1, 2) or presence (lanes 3–8) of HeLa cytosol in the absence (lanes 3, 4) or presence (lanes 5, 6) of ATP-depleting system. The cytosol was preincubated at 4 °C for 90 min without (lanes 3, 4) or with (lanes 7, 8) 10 μl anti-N-terminal antibodies. The cell lysates were either treated with 66 mM Na-citrate buffer, pH 5.0, alone (upper panel) or with 50 mU Endo H (lower panel) in the above buffer at 37 °C for 20 h. R, Endo H resistant form; S, Endo H sensitive form. The amount of transport was calculated as the percentage of HA acquiring Endo H resistance and corrected for the amount of transport in the absence of ATP. *b*, AIF $_{(3-5)}$ treatment does not stimulate the HA transport of HA from the ER to the Golgi. The experiment was done



on intact MDCK cells exactly as described in Fig. 1a except that the 60-min block at 20 °C was skipped. The transport was done at 37 °C for 0 (lanes 1, 7), 5 (lanes 2, 8), 10 (lanes 3, 9), 15 (lanes 4, 10), 25 (lanes 5, 11) or 45 min (lanes 6, 12). The samples were either not treated (upper panel) or treated with Endo H (lower panel) at 37 °C for 20 h as described above.

but not by the C-terminal antibody and the lack of inhibition of ER to Golgi transport by the N-terminal antibody supports the notion that the observed effects are physiologically relevant and not an artefact of the reagents.

We believe that the present observations have two important implications regarding the role of G proteins in membrane trafficking. First of all, these results fulfil the predictions of the G protein model. The effects of AIF $_{(3-5)}$, CTX, MAS and PTX are all consistent with the known behaviour of the G proteins. The mode of action of G proteins in regulating the membrane traffic seems, therefore, similar to that in signal transduction. Thus, it is predicted that there are upstream receptors that relay messages from the luminal side to the G proteins and downstream effectors that regulate unknown aspects of the vesicular transport. Because adenyl cyclase is the best known downstream effector of G $_s$ protein, we wanted to see if the stimulation of apical transport by G $_s$ is mediated by cyclic AMP. Addition of up to 100 μM dibutyl cAMP to cells had no effect on the transport of HA to the apical surface (data not shown). Thus, the effects of G $_s$ on apical transport must be mediated by another downstream effector. The second implication of these studies is that the G proteins may form a part of the sorting machinery in the TGN. The G proteins, with their ability to communicate between both sides of the membrane, are well suited to play this role. Our interpretation of these results is that the G proteins do not affect the vesicular traffic as such but rather regulate the fidelity and/or efficiency of sorting. The more efficient the sorting of a protein in the TGN, the more efficient will be its delivery.

Whatever the mode of G protein action on vesicular trafficking, the intriguing finding is that both G $_s$ and G $_i$ subunits of G proteins seem to regulate the TGN to surface traffic in MDCK cells. Some cells deliver some or all of the apical cargo through the basolateral membrane by a transcytotic pathway¹⁹. There might be a G $_s$ class of G protein involved in the sorting of polymeric IgA receptor during transcytosis²⁰. If so, this demonstrates a remarkable conservation of apical sorting machinery at different locations of epithelial vesicular traffic²¹.

Note added in proof: Recently, Leyte *et al.* (*EMBO J.*, **11**, 4795–4804 (1992)) reported that the G $_s$ class of trimeric G proteins regulate secretory vesicle function in PC12 cells. □

- Higashijima, T., Uzu, S., Nakajima, T. & Ross, E. M. *J. Biol. Chem.* **263**, 6491–6494 (1988).
- Gravotta, D., Adesnik, M. & Sabatini, D. D. *J. Cell Biol.* **111**, 2893–2908 (1990).
- Kobayashi, T., Pimpikar, S. W., Parton, R. G., Bhakdi, S. & Simons, K. *FEBS Lett.* **300**, 227–231 (1991).
- Levis, M. J. & Bourne, H. R. *J. Cell Biol.* **119**, 1297–1307 (1992).
- Rindler, M. J., Ivanov, I. E., Plesken, H., Rodriguez-Boulan, E. & Sabatini, D. *J. Cell Biol.* **98**, 1304–1319 (1984).
- Wandinger-Ness, A., Bennett, M., Antony, C. & Simons, K. *J. Cell Biol.* **111**, 987–1000 (1990).
- Kornfeld, R. & Kornfeld, S. A. *Biochem.* **54**, 631–664 (1985).
- Bartles, J. R., Feracci, H. M., Steiger, B. & Hubbard, A. L. *J. Cell Biol.* **105**, 1241–1251 (1989).
- Bomse, M. & Mostov, K. *J. Cell Biol.* **115**, 195a (1991).
- Simons, K. & Wandinger-Ness, A. *Cell* **62**, 207–210 (1990).
- Matlin, K. & Simons, K. *J. Cell Biol.* **99**, 2131–2139 (1984).
- Sargiacomo, M., Lisanti, M. P., Le Bivic, A. & Rodriguez-Boulan, E. *J. Cell Biol.* **109**, 2117–2127 (1989).

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Infection of natural killer cells by human herpesvirus 6

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NATURAL killer (NK) cells are a functionally defined subset of non-T, non-B lymphocytes of bone marrow origin, which induce lysis of selected target cells, including neoplastic and virus-infected cells^{1–3}. The NK cell function provides an important mechanism of primary defence against viruses *in vivo*, as demonstrated by the occurrence of multiple herpesvirus infections in patients congenitally lacking NK cells^{4,5}. Here we show that functionally competent CD3⁺ NK clones can be productively infected by human herpesvirus 6 (HHV-6)⁶, a T-lymphotropic DNA virus⁷ that may play a role in the acquired immunodeficiency syndrome (AIDS)⁸ and in the chronic fatigue syndrome⁹, two disorders associated with a defective NK cell activity^{10–15}. The infection is cytopathic and induces *de novo* expression of CD4, an antigen not expressed within the NK lineage^{16,17}, thereby predisposing NK cells to infection by human immunodeficiency virus type 1 (HIV-1). These results provide evidence that a herpesvirus can directly target and kill NK cells, a potential strategy to suppress the natural anti-viral immunity of the host.

Susceptibility to HHV-6 infection was initially studied in

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- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **348**, 125–132 (1990).
- Stow, J. L. *et al.* *J. Cell Biol.* **114**, 1113–1124 (1991).
- Barr, F. A. *et al.* *FEBS Lett.* **294**, 239–243 (1991).
- Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J. & Klausner, D. *Science* **254**, 850–853 (1991).
- Colombo, M. I., Mayorga, L. S., Casey, P. J. & Stahl, P. D. *Science* **255**, 1695–1697 (1991).
- Ktistakis, N. T., Linder, M. E. & Roth, M. G. *Nature* **356**, 344–346 (1992).
- Simons, K. & Fuller, S. D. *A. Rev. Cell Biol.* **1**, 243–288 (1985).
- Kahn, R. A. *J. Biol. Chem.* **266**, 15595–15597 (1991).
- Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. & Satoh, T. *A. Rev. Biochem.* **60**, 349–400 (1991).
- Stryer, L. & Bourne, H. R. *A. Rev. Cell Biol.* **2**, 391–420 (1986).
- Higashijima, T., Burnier, J. & Ross, E. M. *J. Biol. Chem.* **265**, 14176–14186 (1990).

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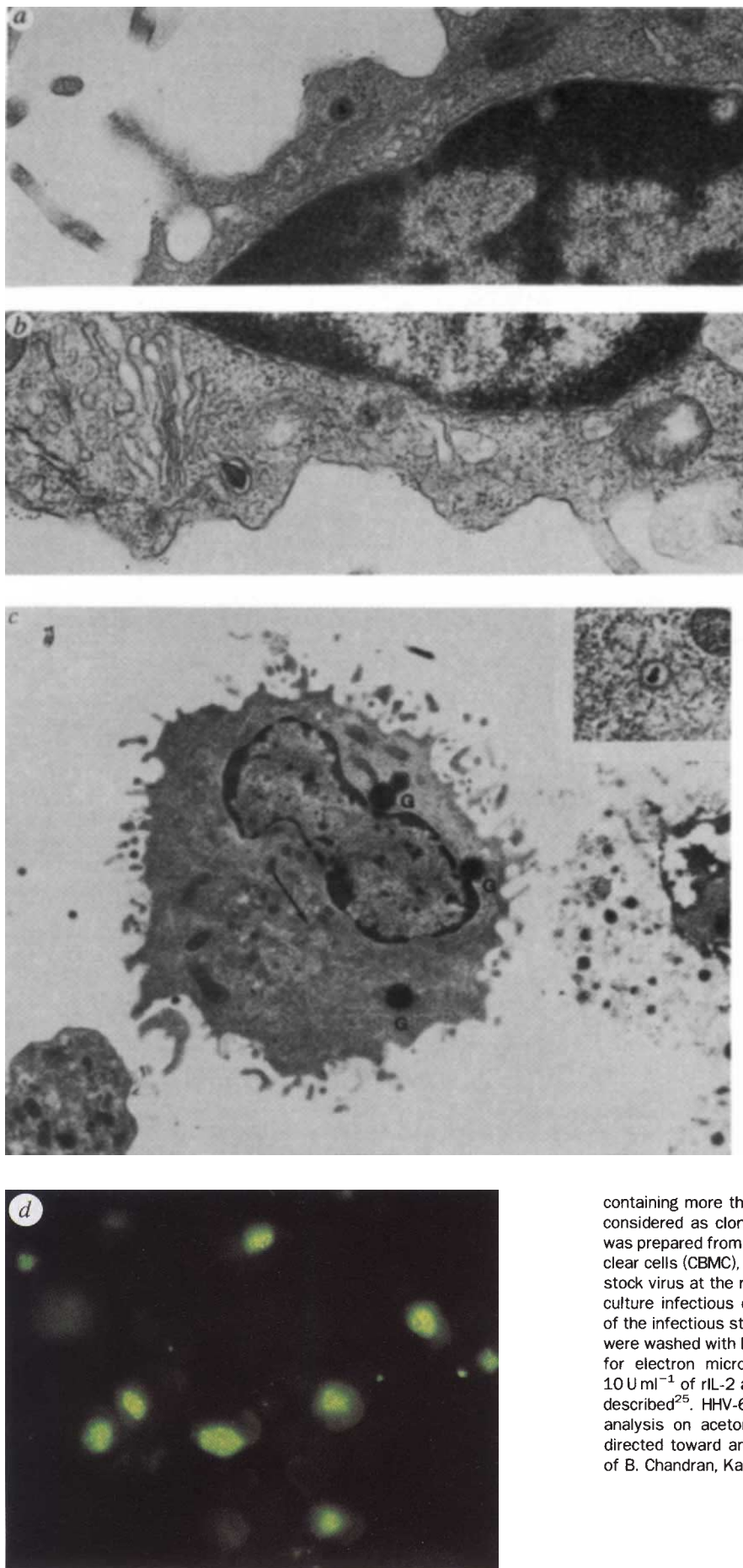


FIG. 1 Infection of human CD3⁺ NK cells by HHV-6. *a, b*, Electron micrographs of freshly isolated, uncultured CD56⁺ NK cells infected with HHV-6 ($\times 60,000$). HHV-6 nucleocapsids are visible within the cytoplasm. Surface membrane CD56 antigens were revealed by the immunogold technique. *c*, Electron micrograph of CD3⁺ NK cell cultured for 14 days with rIL-2 and then infected with HHV-6 ($\times 15,000$), showing the typical large granular lymphocytic morphology (G, cytoplasmic osmiophilic granules). An intracytoplasmic tegument-coated HHV-6 particle and a defective nucleocapsid are indicated by the arrow and magnified ($\times 90,000$) in the insert. Complete and incomplete viral particles are visible in the extracellular space. *d*, Productive HHV-6 infection of a human NK clone (12S/S, non-HHV-6 killer), as detected by indirect IF analysis on acetone-fixed cells. The cells were stained using an antibody directed toward an early-late viral phosphoprotein 3 days after exposure to HHV-6_{GS}. At this stage, the fluorescent signal is mostly restricted to the nucleus of the cells.

METHODS. PBMC were obtained from normal adult volunteers by fractionation over a Ficoll gradient (Nyegaard). CD3⁺ NK cell populations were enriched by negative immunomagnetic selection using a double-rosetting procedure, as previously described⁷. The cells were first labelled with a pool of antibodies (Leu4 (CD3), Leu3a (CD4), LeuM3 (CD14), Leu12 (CD19) and Leu16 (CD20) (Becton-Dickinson), each at $5 \mu\text{g ml}^{-1}$) and subsequently incubated with goat anti-mouse IgG-coated magnetic microspheres (Dyna) at a 40:1 ratio of beads: putative-positive cells. Microsphere-bound cells were then removed with a magnet. Residual T lymphocytes, B lymphocytes or monocytes were consistently less than 1%, determined by IF analysis with the antibodies used for negative selection. The NK cell populations were either used directly for infection studies or stimulated with purified phytohaemagglutinin (PHA) ($0.2 \mu\text{g ml}^{-1}$) and then cultured in the presence of rIL-2 (100 U ml^{-1}). Both freshly isolated and cultured NK cell populations efficiently killed K562, as well as HHV-6-infected, but not uninfected, autologous PMBC. The CD3⁺ NK clones were established by a high-efficiency limiting dilution technique, as previously described²⁷. Briefly, CD3⁺ mononuclear cells from normal adult peripheral blood were seeded at 1 or 3 cells per well in 96-well round-bottom plates (Costar) in the presence of feeder layers of 1×10^5 autologous, 4,000 rad-irradiated PBMC, purified PHA at $0.2 \mu\text{g ml}^{-1}$ and rIL-2 at 100 U ml^{-1} . After 6 days, $100 \mu\text{l}$ of medium from each well were replaced with $100 \mu\text{l}$ of complete medium containing 1×10^5 irradiated autologous PBMC and rIL-2. When the cells reached a satisfactory number, they were phenotypically characterized. Only wells

containing more than 99.0% cells with a CD3⁺ 4⁺ 16⁺ 56⁺ phenotype were considered as clones and used for subsequent studies. The HHV-6 stock was prepared from infected cultures of human umbilical cord blood mononuclear cells (CBMC), as described⁷. Pelleted NK cells were incubated with the stock virus at the rough multiplicity of infection (MOI) of 1 (that is, 1 tissue culture infectious dose per cell, as determined by serial 10-fold dilutions of the infectious stock on PHA-activated CBMC). After 1 h at 37°C , the cells were washed with PBS and then either labelled with Leu19 (CD56) and fixed for electron microscopy or resuspended in culture medium containing 10 U ml^{-1} of rIL-2 and cultured at 37°C . Immunogold labelling was done as described²⁵. HHV-6 infection was monitored thrice weekly by indirect IF analysis on acetone-fixed cells, as described⁷, using antibody 9A5D12, directed toward an early-late viral phosphoprotein of *M*, 41/110K (a gift of B. Chandran, Kansas City, KN)²⁸.

freshly isolated human CD3⁺ NK cell populations purified from the peripheral blood of healthy adult volunteers. These cells efficiently lysed the erythroleukaemic cell line K562, as well as autologous peripheral blood mononuclear cells (PBMC) infected with HHV-6. After exposure to HHV-6 strain GS (ref. 6) NK cells were either cultured in the presence of recombinant human interleukin-2 (rIL-2) or labelled by the immunogold technique with an anti-CD56 monoclonal antibody and examined by electron microscopy. Freshly isolated CD56⁺ NK cells readily internalized HHV-6 particles (Fig. 1a, b), but no signs of viral replication were observed, even after long-term culture (>30 days). Analogous results were obtained with NK cell populations cultured for 10–14 days before infection (Fig. 1c). By contrast, productive infection was detected when NK cells were cultured *in vitro* for more than 5 weeks before exposure to HHV-6. Because long-term culturing results in a progressive loss of NK cytolytic activity against HHV-6-infected targets (M.M., unpublished data), these data suggested that NK cells are indeed infectable by HHV-6, although viral replication is suppressed by functional anti-viral effectors.

By extensive clonal analysis, we have demonstrated that circulating NK cells encompass functionally heterogeneous effectors: although all CD3⁺56⁺ NK clones lyse K562, only a proportion of them efficiently kill autologous PBMC infected by HHV-6 (M.M. *et al.*, manuscript in preparation). This latter phenotype will be referred to as 'HHV-6 killer' phenotype. We hypothesized that HHV-6 might replicate unimpeded in NK clones lacking the ability to kill autologous infected targets. To test this hypothesis, five NK clones with an HHV-6 killer phenotype and seven with a non-HHV-6 killer phenotype (Table 1) were studied for their susceptibility to infection by HHV-6_{GS}. The clones contained more than 99.0% CD3⁺4⁺16⁺56⁺ cells and killed K562 with high efficiency. By day 7–10 after exposure to HHV-6, signs of productive infection were found by immunofluorescence (IF) in all the clones with the non-HHV-6 killer phenotype (Fig. 1d). The infection was associated with the appearance of typical HHV-6-induced cytomorphological changes and resulted in the complete extinction of the cultures within 10–12 days. In contrast, virus replication was not detectable in any of the HHV-6 killer clones. These results are consistent with the concept that HHV-6 replication is suppressed in NK cell cultures which recognize HHV-6-infected cells as targets.

During the course of HHV-6 infection, the NK clones were monitored by fluorocytometry for CD3, CD4 and CD56 expression. A dramatic induction of CD4 was observed in infected clones (Fig. 2a). Precise identification of CD4 was achieved using three different antibodies (Leu3a, OKT4, OKT4a) that recognize distinct epitopes on two domains of the glycoprotein. A slight downregulation of CD56 was seen in a limited proportion of infected cells, whereas CD3 remained negative after infection. Unlike CD3⁺8⁺ T lymphocytes, CD3⁺ NK cells undergo an extrathymic development and do not express CD4 at any stage during their differentiation. Previous evidence for the expression of CD4 by NK cells is limited to a single report of a weak positivity in 1 out of more than 500 CD3⁺GL183⁺ NK clones screened¹⁸.

To investigate at which level the expression of CD4 was regulated, CD4 messenger RNA was assayed in two NK clones infected by HHV-6 and their uninfected counterparts. Owing to the limited number of cells available, the analysis was done by polymerase chain reaction (PCR) amplification of reverse transcribed total cellular RNA (Fig. 2b). As expected, no signal was detected in uninfected clones (lanes 4, 6) despite the high sensitivity of the test. Instead, CD4 mRNA was induced in both NK clones following HHV-6 infection (lanes 5, 7). These data indicate that in NK cells also, as previously described in CD3⁺8⁺ T lymphocytes¹⁹, HHV-6 activates CD4 expression at the transcriptional level.

Several NK clones of different phenotype were also studied for their susceptibility to HIV, the causative agent of AIDS. No

TABLE 1 Phenotypic and functional characteristics of the NK clones used for HHV-6 infection

Clone	CD3	CD4	CD16	CD56	K562	PBMC*	Specific lysis (%) of
							HHV-6 ⁺ PBMC†
3S46R	—	—	+	+	81.7	0.0	54.0
9S46P	—	—	+	+	85.2	0.0	33.7
9S46R	—	—	+	+	98.9	3.0	23.2
A22/3	—	—	+	+	66.7	0.0	26.0
A31/6	—	—	+	+	73.6	0.0	35.1
6D5.1	—	—	+	+	75.1	0.0	0.0
8S46P	—	—	+	+	90.4	0.0	8.9
12S/S	—	—	+	+	84.3	0.0	4.1
12S46P	—	—	+	+	84.9	0.0	7.3
15S46P	—	—	+	+	74.5	0.0	5.4
19S/S	—	—	+	+	86.2	0.0	2.9
25/D5	—	—	+	+	84.4	NT	5.7

Surface membrane antigen expression was analysed by fluorocytometry, as described in the legend to Fig. 2. For cytotoxicity studies, a standard ⁵¹Cr-release test was used, as described in detail elsewhere²⁷. The per cent specific lysis refers to an 8:1 effector:target ratio and was calculated according to the following formula: (sample c.p.m. – spontaneous c.p.m.)/maximal c.p.m. – spontaneous c.p.m.) × 100. HHV-6 killer clones were defined as those exhibiting more than 10.0% specific lysis against autologous HHV-6-infected cells at 8:1 effector:target ratio.

* Autologous PBMC activated with purified PHA (1 µg ml⁻¹).

† Autologous PBMC previously activated with PHA and subsequently infected with HHV-6_{GS} at the rough multiplicity of infection (MOI) of 1. Virus replication was detected in more than 80% of the target cells before ⁵¹Cr labelling.

NT, Not tested.

signs of productive infection were detected in any of the clones (data not shown). We hypothesized that the ability of HHV-6 to induce *de novo* expression of CD4, the major receptor for HIV²⁰, could predispose NK cells to HIV-1 infection. To explore this possibility, a CD4⁺ NK clone was infected with HHV-6 and subsequently exposed to HIV-1, strain IIIB. Significant levels of HIV-1 p24 antigen, indicative of HIV-1 replication, were released by NK cells previously infected with HHV-6 (Fig. 3). In contrast, no detectable p24 antigen was produced by homologous HHV-6 negative cells. Infection by HIV-1 was totally abrogated by pretreatment of the cells with OKT4a, an HIV-neutralizing anti-CD4 antibody. These results demonstrate that, by inducing *de novo* expression of CD4, HHV-6 renders NK cells susceptible to infection by HIV-1. In contrast to our results, other investigators have reported that NK cells can be directly infected by HIV-1 in the absence of CD4^{21–23}. In two studies^{21,22}, however, the presence of residual CD4⁺ cells within the NK cell preparations could not be ruled out. In a third report²³, highly purified NK cell populations, previously exposed to irradiated Epstein-Barr virus (EBV)-transformed B cells, were infected, albeit with low efficiency, by HIV-1. The reasons for the discrepancies between this and our study are unclear. It is possible that uncloned NK cell populations encompass infrequent cells which can be infected by HIV-1 in a CD4-independent fashion or, alternatively, that coculture with EBV-immortalized B cells may induce alterations in the physiology of NK cells.

Several strategies allow viruses to elude the surveillance of the immune system and establish persistent infection in the host. One such mechanism is the immunosuppression caused by direct infection and functional impairment of immune cells²⁴. To our knowledge, HHV-6 is the only virus hitherto described that efficiently infects and kills NK cells. HHV-6 is a potentially immunosuppressive agent, as suggested by its tropism for both CD4⁺ and CD8⁺ T cells^{7,19} and its ability to downregulate CD3²⁵, a crucial molecule in the physiology of immunocompetent T lymphocytes. A reduced NK cell function has been

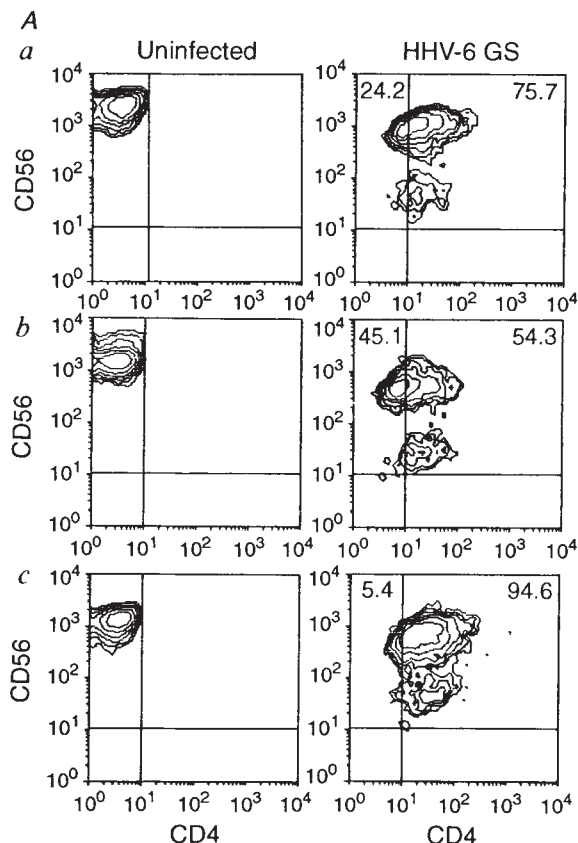
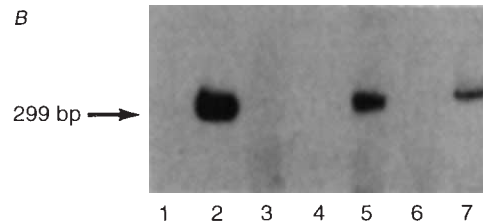


FIG. 2 Induction of *de novo* CD4 expression in NK cells by HHV-6. **A**, Two-colour fluorocytometric analysis of CD56 and CD4 expression in three representative CD3⁺ NK clones infected by HHV-6 and their uninfected counterparts. **a**, Clone 15S46P; **b**, clone 8S46P; **c**, clone 12S46P. All the clones efficiently killed the K562 cell line, a classical NK target, but not autologous HHV-6-infected PBMC. **b**, Induction of CD4 mRNA in NK clones infected with HHV-6, as detected by PCR amplification of reverse transcribed mRNA and subsequent Southern blot analysis. Lane 1: PCR reagents (negative control); lane 2: unfractionated normal human PBMC (more than 70% CD4⁺); lane 3: human B-lymphoblastoid cell line Raji (CD4⁺); lane 4: uninfected NK clone 12S46P; lane 5: HHV-6-infected 12S46P; lane 6: uninfected NK clone 8S46P; and lane 7: HHV-6-infected 8S46P.

FIG. 3 CD4-dependent HIV-1 infection of NK cells previously infected by HHV-6. NK clone 12S/S previously uninfected (open bars); 12S/S preinfected with HHV-6 and treated with antibody OKT4a (stripped bars); 12S/S preinfected with HHV-6 (solid bars). Infection with HIV-1_{IIIB} was done 48 h after exposure to HHV-6, at a time when *de novo* CD4 expression was already detectable on the cell surface membrane. The HIV-1 stock was grown in the continuous cell line H9. Infection was done for 1 h at 37 °C at the rough MOI of 1 (as determined by serial 10-fold dilutions of the virus stock on H9 cells). The cells were then washed and cultured in the presence of rIL-2. To minimize the amount of residual virus from the HIV-1 inoculum, the cells were washed four times in a 15 ml volume of culture medium, each time carefully removing all the residual medium after pelleting. HIV-1 replication was monitored by measuring the amount of HIV-1 p24 Gag protein in the culture supernatants using a commercial antigen capture ELISA (Dupont). Antibody OKT4a (Ortho), previously dialysed and sterilized by filtration, was used to pretreat the cells at 5 $\mu\text{g ml}^{-1}$ and maintained within the culture at the same concentration thereafter.

documented in chronic fatigue syndrome (CFS)¹³⁻¹⁵, a complex disorder of unknown aetiology²⁶, often presenting with signs of active HHV-6 infection⁹. Multiple lines of evidence suggest that HHV-6 may act as a cofactor in AIDS⁸, another syndrome associated with a marked functional impairment of the NK cell activity¹⁰⁻¹². We have demonstrated that HHV-6 is not only directly infectious and cytopathic for NK cells, but also induces them to express CD4, thus acquiring susceptibility to HIV-1



B

299 bp →

1 2 3 4 5 6 7

METHODS. The antibodies used for fluorocytometric analysis were fluorescein isothiocyanate-conjugated Leu3a and phycoerythrin-conjugated Leu19. Other antibodies used to characterize the cells were OKT4, OKT4a (Ortho Diagnostics), Leu2a (CD8), and Leu11a (CD16) (Becton-Dickinson). Infection was done as described in the legend to Fig. 1. The distribution of surface antigens was studied using a FacScan analyser (Becton-Dickinson). In infected cultures, dead cells were removed before the analysis by centrifugation on Ficoll-Hypaque gradient (Nyegaard). Negative controls were stained with mouse IgG₁ irrelevant antibodies conjugated with either fluorochrome. For mRNA analysis, infected NK clones were collected at day 8 after exposure to HHV-6. Uninfected clones were cultured in parallel and collected at the same time. Total cellular RNA was extracted as previously described²⁹. An aliquot of 250 ng of RNA was incubated at 37 °C for 30 min with 30 pmol of antisense primer (CD4.2), 100 μM each dNTP (Pharmacia), 1 mM DTT (Promega) and 24 U of AMV reverse transcriptase (RT) (Pharmacia) diluted in the same buffer used for PCR amplification (see below), in a total volume of 20 μl . A 5 μl aliquot of each RT reaction was subjected to 30 cycles of PCR (94 °C for 1 min, 58 °C for 2 min, 72 °C for 2 min, with an extension of 2 s of the third step at each cycle, plus 10 min of further extension at the end of the process) in the presence of 500 μM of each dNTP (Perkin-Elmer Cetus, Norwalk, CT), 50 pmol of each primer, 10 U of *Taq* Polymerase (Perkin-Elmer Cetus), 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , in a total volume of 100 μl . Mineral oil (50 μl) was loaded on top of the reaction mixture. Of each sample, 20 μl were subsequently electrophoresed in a 6% polyacrylamide gel at 200 V for 2 h in TBE buffer and then electroblotted for 2 h in the same buffer at 30 V onto a Nylon membrane (BioRad). The membrane was crosslinked by ultraviolet irradiation, prehybridized at 42 °C for 2 h in 6 $\times$ SSC, 5 $\times$ Denhardt solution, 100 $\mu\text{g ml}^{-1}$ single-stranded DNA and hybridized overnight in the same buffer at 42 °C with ³²P-labelled probe. The membrane was then washed in 3 $\times$ SSC, 0.1% SDS at room temperature for 20 min and at 37 °C for 20 min. The autoradiogram was exposed for 2 h at -70 °C. The primers used for amplification were:

CD4.1 5'-GCAGTGGCGAGCTGTGGT-3';

CD4.2 5'-GGGTCCCCACACCTCACAGG-3'.

The probe (CD4.3) was:

5'-GAACCTGGTGGTGTGATGAGAGCCACTCAGGT-3'.

infection. These results elucidate a novel mechanism whereby HHV-6 may contribute to the immune dysfunctions associated with CFS and AIDS. *In vivo* studies will be critical to establish the role of HHV-6 definitively in these disorders. □

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- Herberman, R. B. & Ortaldo, J. R. *Science* **214**, 24-27 (1981).
- Trinchieri, G. *Adv. Immun.* **47**, 187-211 (1989).

3. Robertson, M. J. & Ritz, J. *Blood* **76**, 2421-2438 (1991).
4. Fleisher, G., Koven, N., Kamiya, H. & Henle, W. *J. Pediatr.* **100**, 727-733 (1982).
5. Biron, C. A., Byron, K. S. & Sullivan, J. L. *N. Engl. J. Med.* **320**, 1731-1734 (1989).
6. Salahuddin, S. Z. *et al. Science* **234**, 596-601 (1986).
7. Lusso, P. *et al. J. exp. Med.* **167**, 1659-1670 (1988).
8. Lusso, P., Ablashi, D. V. & Luka, J. in *Human Herpesvirus-6* (eds Ablashi, D. V., Krueger, G. R. F. & Salahuddin, S. Z.) 121-133 (Elsevier, Amsterdam, 1992).
9. Buchwald, D. *et al. Ann. Intern. Med.* **116**, 103-113 (1992).
10. Rook, A. H. *et al. J. clin. Invest.* **72**, 398-403 (1983).
11. Bonavida, B., Katz, J. D. & Gottlieb, M. *J. Immun.* **137**, 1157-1160 (1986).
12. Sirianni, M. C., Sodd, S., Malorni, W., Arancia, G. & Aiuti, F. *J. Immun.* **140**, 2565-2568 (1988).
13. Aoki, T., Usuda, Y., Miyakoshi, H., Tamura, K. & Herberman, R. B. *Nat. Immun. Cell Growth Regul.* **6**, 116-128 (1987).
14. Caligiuri, M. *et al. J. Immun.* **139**, 3306-3313 (1987).
15. Klimas, N. G., Salvato, F. R., Morgan, R. & Fletcher, M. A. *J. clin. Microbiol.* **28**, 1403-1410 (1990).
16. Lanier, L. L. & Phillips, J. H. in *Leukocyte Typing II* Vol. 3 (eds Reinherz, E. L., Haynes, B. F., Nadler, L. M. & Bernstein, I. D.) 157-198 (Springer, New York, 1986).
17. Ritz, J. *et al. Adv. Immun.* **42**, 181-211 (1988).
18. Moretta, A. *et al. J. exp. Med.* **171**, 695-714 (1990).
19. Lusso, P. *et al. Nature* **337**, 370-373 (1991).
20. Dalglish, A. G. *et al. Nature* **312**, 763-767 (1984).
21. Ruscetti, F. W. *et al. J. Immun.* **136**, 3619-3624 (1986).
22. Robinson, W. E. *et al. Hum. Pathol.* **19**, 535-540 (1988).
23. Chehimi, J. *et al. J. Virol.* **65**, 1812-1822 (1991).
24. Oldstone, M. B. A. *J. Virol.* **65**, 6381-6386 (1991).
25. Lusso, P. *et al. J. Immun.* **147**, 2147-2153 (1991).
26. Holmes, G. P. *et al. Ann. intern. Med.* **108**, 387-391 (1988).
27. Moretta, A., Pantaleo, G., Moretta, L., Cerottini, J. C. & Mingari, M. C. *J. exp. Med.* **157**, 743-751 (1983).
28. Balachandran, N., Amelse, R. E., Zhou, W. W. & Chang, C. K. *J. Virol.* **63**, 2835-2840 (1989).
29. Chomczynski, P. & Sacchi, M. *Analyt. Biochem.* **162**, 156-159 (1987).

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Oncogene *ect2* is related to regulators of small GTP-binding proteins

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WE have developed an efficient expression cloning system that allows rapid isolation of complementary DNAs able to induce the transformed phenotype^{1,2}. We searched for molecules expressed in epithelial cells and possessing transforming potential to fibroblasts, and cloned a cDNA for the normal receptor of a growth factor secreted by NIH/3T3 cells^{3,4}. Here we report a second novel transforming gene, *ect2*. The isolated cDNA is activated by amino-terminal truncation of the normal product. The Ect2 protein has sequence similarity within a central core of 255 amino acids with the products of the breakpoint cluster gene, *bcr* (ref. 5), the yeast cell cycle gene, *CDC24* (ref. 6), and the *dbl* oncogene⁷. Each of these genes encodes regulatory molecules or effectors for Rho-like small GTP-binding proteins⁸⁻¹⁰. The baculovirus-expressed Ect2 protein could bind highly specifically to Rho and Rac proteins, whereas the *dbl* product showed broader binding specificity to Rho family proteins. Thus *ect2* is a new member of an expanding family, whose products have transforming properties and interact with Rho-like proteins of the Ras superfamily.

An *ect2*-transformed focus was induced after transfection of NIH/3T3 cells with a BALB/MK mouse keratinocyte expression cDNA library in λ pCEV27 (ref. 2), and genomic DNA from the transformed cells was subjected to plasmid rescue³. A plasmid, designated p3N-11, had high-titred transforming activity on transfection of NIH/3T3 cells. The *ect2* foci exhibited an unusual stellate morphology containing both fusiform and rounded cells (Fig. 1a). A mass population of marker-selected *ect2*-transfected cells formed rapidly growing colonies at high efficiency (1-10%) in semi-solid agar medium, another *in vitro* characteristic of transformed cells. Subcutaneous inoculation of nude mice with 10^6 *ect2* cells led to palpable tumours in 100% of animals within 6 weeks, whereas marker-selected control NIH/3T3 cells failed to form any tumours, demonstrating that *ect2* transfectants exhibited the malignant phenotype.

Hybridization of the 2.8 kilobase (kb) *ect2* cDNA insert with poly(A)⁺ RNA from BALB/MK cells revealed a single 4.0 kb transcript. The transcript was also detectable in kidney, liver, spleen and, at highest levels among the tissues analysed, in testis (Fig. 1b). By screening the BALB/MK cDNA library with the p3N-11 insert as probe, we isolated additional cDNA clones,

whose sizes closely corresponded to the 4.0 kb transcript. Sequence analysis of one of these 4.0 kb cDNAs, designated CL7, revealed a long open reading frame of 2,214 base pairs (bp) encoding a predicted 84K protein (Fig. 2a). Comparison of the sequence of the p3N-11 clone with CL7 revealed that p3N-11 was truncated at its N terminus, beginning at amino-acid residue 212 in the complete *ect2* coding sequence. There was no match with any known gene in the GenBank and EMBL nucleotide and SWISS-PROT protein sequence databases. But

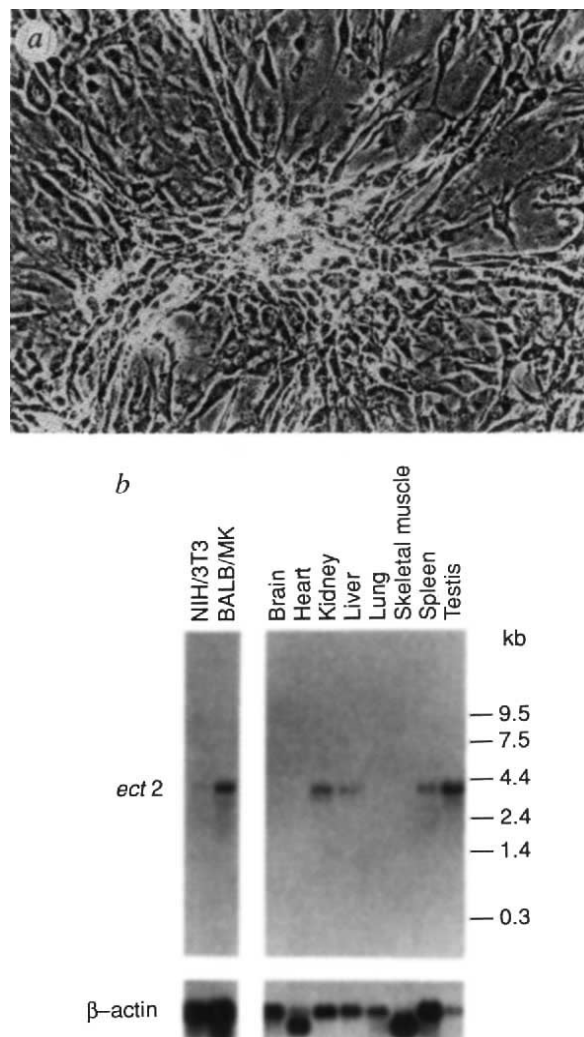
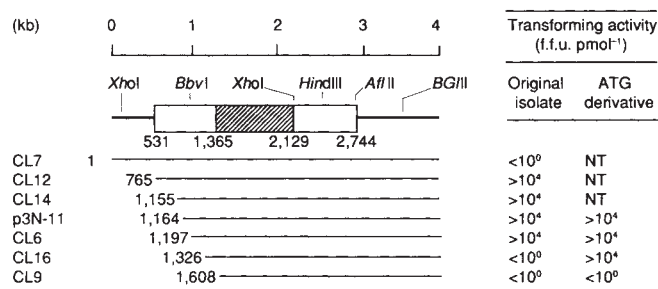


FIG. 1 Expression of the *ect2* gene. a, Cell morphology of the transformed foci induced by the *ect2* expression vector at 21 day post-transfection ($\times 180$). b, Northern analysis of poly(A)⁺ RNAs from NIH/3T3 and BALB/MK cells and different mouse tissues. RNAs were fractionated, blotted and probed with (top) *ect2* cDNA insert from p3N-11 or (bottom) β -actin cDNA as described previously³. Molecular size markers are shown in kb.

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FIG. 3 Schematic representation of the *ect2* cDNA clones and their transforming activities. Untranslated regions are shown by thick lines and the coding sequence is shown by a box. Hatched portion indicates the DH domain. Positions of nucleotides that delimit the domains are shown under the coding region box. The region carried by each clone is shown by a line and the nucleotide position of each 5'-end in the *ect2* CL7 sequence is indicated (see Fig. 2a). f.f.u., Focus-forming units; NT, not tested.

METHODS. The first *ect2* plasmid, p3N-11, was rescued from transformed cells of the *ect2* focus. Other cDNA clones were isolated by screening of the BALB/MK library with p3N-11 cDNA insert as a probe. Plasmids with the same structures as p3N-11 and CL6 were also identified in two other *ect2* foci induced by the BALB/MK cDNA library. To add a consensus translation initiation site at the 5'-end of the truncated cDNA clones, oligonucleotides were inserted between the *Bam*HI site (in the vector) and the *Nru*I site (in the *Sfi*I adaptor²) located upstream from the cDNA sequence. The resulting plasmids (ATG derivatives) were sequenced to confirm the presence of the ATG codon in-frame with the truncated *ect2* coding sequence. The ATG oligonucleotides had following sequences: 5'-GATCCTAGGCCACCATG-



GAAATCG-3', 5'-CGATTTCATGGTGGCCTAG-3'. Transformed foci were scored 21 days after transfection. In all of the transfection experiments, more than 10⁴ of G-418-resistant colonies were formed per pmol of each plasmid DNA. Two other 4-kb *ect2* clones similar to CL7 did not show detectable transforming activity.

the predicted Ect2 protein contained a central domain consisting of 255 residues, which had sequence similarity with analogously located domains within the *bcr*, *CDC24*, and *dbf* gene products¹¹ (Fig. 2b). The core region of dbf-homology (DH) domain (amino acids 281-465) of *ect2* showed 31%, 22% and 24% identity, respectively, with those of Bcr, CDC24 and Dbf proteins. A chromosomal rearrangement of *bcr* and *abl* is involved in the

activation of the *bcr-abl* oncogene in chronic myelogenous leukaemia (CML)⁵. *CDC24* is a yeast cell cycle gene associated with defective budding⁶, whereas NIH/3T3 transfection of a human tumour DNA led to identification of the *dbf* transforming gene⁷. No other similarities were detected between the *ect2* product and any of these molecules.

To investigate the mechanism involved in activation of trans-

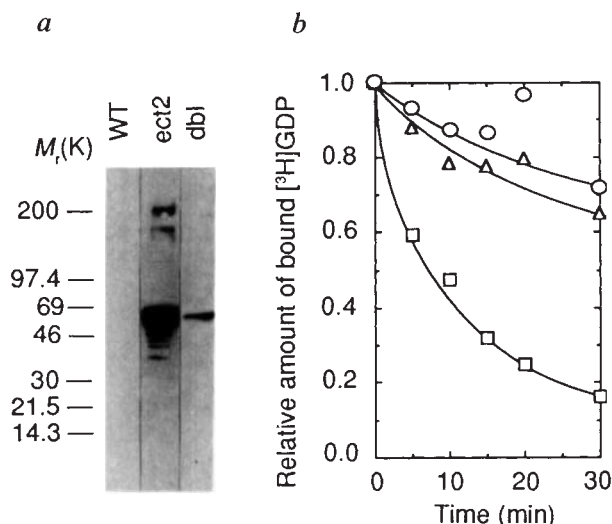
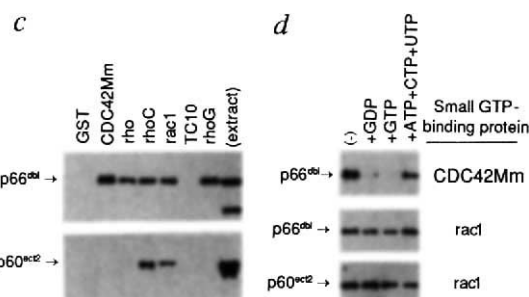


FIG. 4 Expression and functional analysis of the Ect2 protein. *a*, Production of the FLAG epitope-tagged Ect2 protein in insect cells. Soluble fractions (20 μ l each) of *S. frugiperda* (Sf9) cells infected with wild-type (WT), FLAG-Ect2-expressing (ect2), or Dbf-expressing (dbf) baculovirus were analysed by immunoblotting using anti-FLAG M2 monoclonal antibody (IBI, New Haven) for WT and ect2, or anti-dbf antisera raised against a synthetic peptide¹⁰ for dbf. The bands were visualized by ECL detection system (Amersham). Locations of molecular mass markers are at the left. *b*, Effects of the Sf9 cell extracts containing Ect2 protein on [³H]GDP dissociation from the murine CDC42 protein. Extracts (8 μ l) of Sf9 cells infected with the wild-type virus (circles), FLAG-Ect2 virus (triangles), or Dbf virus (squares) were added to incubations (32 μ l) containing the murine CDC42 protein (CDC42Mm) and the time courses for the dissociation of [³H]GDP (Amersham; 12.6 Ci mmol⁻¹) from the GTP-binding protein were measured as described¹⁰. The assay was done several times under similar conditions and a typical result is shown. *c*, Binding of Dbf and Ect2 proteins to Rho family small GTP-binding proteins. Binding assays were done as described²⁰. The baculovirus-infected Sf9 cell extracts were dialysed and precleared with GST-beads. Aliquots (0.1 ml) were mixed with GST-beads or GST-Rho family protein beads (indicated on top) to precipitate the Dbf or Ect2 protein. The samples, in addition to the precleared lysates (10 μ l each), were analysed as in *a*, except that affinity-purified anti-dbf rabbit polyclonal antibodies (Santa Cruz Biotechnology, CA) were used to detect Dbf. The lower band in the Dbf extract seems to be a degradation product of p66^{dbf}. *d*, Effect of



the presence of guanine nucleotides on binding. Binding assays were done in the absence (leftmost lanes) or presence (other lanes) of 0.5 mM of GDP, GTP, or mixture of ATP, CTP and UTP, as indicated, and processed as in *c*. The Rho family proteins used to precipitate Ect2 or Dbf are shown at the right. METHODS. Oligonucleotides were inserted at the 5'-end of the *ect2* cDNA insert of p3N-11 (see Fig. 3) to add the FLAG epitope of 8 amino acids²⁴ followed by an ATG codon at the N terminus of the protein. The ATG-FLAG-*ect2* plasmid showed a similar focus formation efficiency on NIH/3T3 cells as p3N-11, indicating that the fusion protein is functionally active. The ATG-FLAG-*ect2* insert was then subcloned in a baculovirus expression plasmid pVL941, and the DNA was used to transfect Sf9 cells with linearized baculovirus DNA using BaculoGold transfection kit (PharMingen, San Diego, CA). The Sf9 cells were infected with the wild-type or *ect2*-recombinant virus and collected 60 h after infection. The *dbf*-recombinant virus-infected cells has been described¹⁰. These infected cells were sonicated in an extraction buffer¹⁰ and the supernatants obtained after centrifugation for 15 min at 4 $^{\circ}$ C were used in all the experiments. The small GTP-binding proteins were cloned by polymerase chain reaction (PCR) in pCEV30G (T.M., unpublished data), a bacterial expression plasmid based on the T7 promoter²⁵, to fuse the proteins in frame with GST. The entire sequence of each insert was determined. The mouse *CDC42* clone (*CDC42Mm*) was 95% identical to the human clone (*CDC42Hs*)^{13,14} in nucleotide level, and their amino-acid sequences were completely identical. Other clones were isolated from human cDNA libraries and their sequences were as reported except follows. Nucleotides A, C, G and A in positions 526, 564, 567 and 570 in the published *rhoG* sequence²⁶ were found to be G, G, C and G, respectively in our clone. Our sequence agrees to the hamster *rhoG* cDNA sequence²⁶ in these positions. The nucleotide position 452 of the published *rho* sequence²⁷ was found to be T in our sequence. These proteins were expressed²⁵ and purified²⁸ as described. The CDC42Mm protein was prepared by thrombin cleavage of the GST-CDC42Mm fusion protein followed by removal of the GST moiety²⁸.

forming activity, we compared biological activity of p3N-11 with the full-length *ect2* cDNA as well as other clones isolated by screening the BALB/MK cDNA library. Figure 3 shows that the 4.0 kb *ect2* cDNA exhibited no detectable transforming activity, whereas the truncated clones (CL12, CL14, p3N-11 and CL6) induced greater than 10^4 focus-forming units per pmol under the same assay conditions. These results indicated that truncation of its N-terminal region was involved in the activation of *ect2* transforming function.

Analysis of CL16 and CL9 revealed the extent to which N-terminal truncation could be done without loss of transforming function. Although neither of these clones showed detectable biological activity, both lacked a strong consensus translation initiator. Therefore we inserted oligonucleotides containing a consensus initiator at the 5' ends of these clones as well as p3N-11 and CL6. The ATG-derivative of CL16 exhibited high-titred transforming activity, similar to that of p3N-11. In contrast, CL9 remained nontransforming. These results suggested that deletion of an additional 96 residues including the first 81 amino acids of the conserved domain was sufficient to inactivate *ect2* biological function.

The central core of *ect2* gene product has significant homology with the Bcr, CDC24 and Dbl proteins. These proteins have been implicated as regulators or effectors of Rho-like products of the Ras superfamily of small GTP-binding proteins. A stretch of 29 amino acids near the C terminus of the *ber* protein shows similarity to a purified GTPase activating protein (GAP) for p21^{rho} and another GAP, *n-chimaerin*⁸. Moreover, the bacterially expressed Bcr protein C terminus exhibits *in vitro* GAP activity for p21^{rac} (ref. 8). The defect in yeast budding associated with *cdc24* is shared by another mutant, *cdc42* (ref. 12), whose human homologue *CDC42Hs* encodes a Rho-related small GTP-binding protein^{13,14}. The *cdc24* lesion can be suppressed by overexpression of *CDC42*, implying that *CDC24* may be a regulator of this GTP-binding protein⁹. The baculovirus-infected insect cell extract containing *dbl* oncogene product catalyses GDP/GTP exchange on *CDC42Hs* *in vitro*¹⁰. To test whether Ect2 protein can also act as an exchange factor towards *CDC42*, we cloned the murine *CDC42* cDNA and purified the protein. The transforming Ect2 protein was expressed in a baculovirus expression system¹⁵ (Fig. 4a), and the extract of the virus-infected cells was used to assay for guanine nucleotide exchange activity. The Ect2 protein-containing extract did not catalyse GDP/GTP exchange on the purified *CDC42Mm* protein, whereas the Dbl protein-containing extract possessed this activity, although it was much lower than the reported activity on the *CDC42Hs* protein purified from human platelet¹⁰ (Fig. 4b). We also could not detect exchange activity in the Ect2 extract on Rho and Rac1 proteins expressed as glutathione *S*-transferase (GST) fusion proteins (data not shown). These results suggested that the Ect2 protein alone had no catalytic

activity as an exchange factor on the Rho family small GTP-binding proteins under these conditions. The Ect2 protein may have exchange activity for other small GTP-binding proteins, or possibly modulate the activity of small GTP-binding proteins by an unknown mechanism.

A small GTP-binding protein, Ran, associates with its cognate exchange factor, RCC, and this complex dissociates in the presence of excess GDP or GTP¹⁶, suggesting that regulators of small GTP-binding proteins can be identified by their specific binding. To test whether the Ect2 protein can interact with Rho family small GTP-binding proteins, we expressed and purified several more Rho family small GTP-binding proteins in addition to *CDC42Mm* as GST fusion proteins and examined specific binding to the baculovirus-expressed Ect2. The Ect2 protein showed specific binding to RhoC and Rac1 and, with less efficiency, to Rho (Fig. 4c). In contrast, Dbl protein showed specific binding to all of these Rho family proteins except TC10. The defective binding of Dbl protein to TC10 may be explained by possible N-terminal truncation of the TC10 protein, because this protein was expressed from the third methionine codon of the reported cDNA sequence¹⁷. In the presence of excess GDP or GTP, the specific binding of Dbl protein to *CDC42Mm* was drastically reduced, whereas the presence of other nucleotides had little effect (Fig. 4d). These binding properties were consistent to the exchange activity of Dbl protein on *CDC42Mm*. In contrast to *CDC42Mm*, Rac1 could form a complex with either Dbl or Ect2 protein (Fig. 4c). These complexes were detected even in the presence of guanine nucleotides (Fig. 4d). Because only a weak GDP/GTP exchange activity of Dbl protein on Rac1 was reported¹⁰ and we could not detect exchange activity of Ect2 protein on Rac1, this guanine nucleotide-insensitive binding may be attributed to another function rather than guanine nucleotide exchange. Detailed mapping is needed to find specific binding sites of these proteins, but DH domains may be involved in binding to Rho family proteins. Our findings strongly suggest that Ect2 protein can interact with and may modulate the activity of Rho and Rac proteins and/or possibly closely related unknown small GTP-binding proteins.

Although members of the Bcr/CDC24/Dbl protein family share common features, each has different structural motifs and functional activities. For example, the *CDC24* protein can bind Ca^{2+} ions¹⁸, whereas the *ber* product has serine/threonine-kinase activity¹⁹ as well as a motif which allows binding to the SH2 domain of the *abl* oncogene product²⁰. Recently two other molecules with DH domains were reported; Vav, an oncogene product which contains SH2 and SH3 domains²¹, and Ras-GRF, a GDP/GTP exchange factor for Ras proteins²². Further characterization of *ect2* and others of this family should help in elucidating signal transduction mediated by the small GTP-binding proteins as well as aberrations in these pathways associated with oncogenic transformation. □

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- Miki, T., Matsui, T., Heidaran, M. A. & Aaronson, S. A. *Gene* **83**, 137-146 (1989).
- Miki, T. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5167-5171 (1991).
- Miki, T. et al. *Science* **251**, 72-75 (1991).
- Miki, T. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 246-250 (1992).
- Hariharan, I. K. & Adams, J. M. *EMBO J.* **6**, 115-119 (1987).
- Hartwell, L. H., Mortimer, R. K., Culotti, J. & Culotti, M. *Genetics* **74**, 267-286 (1973).
- Eva, A. & Aaronson, S. A. *Nature* **316**, 273-275 (1985).
- Diekmann, D. et al. *Nature* **351**, 400-402 (1991).
- Bender, A. & Pringle, J. R. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9976-9980 (1989).
- Hart, M. J., Eva, A., Evans, T., Aaronson, S. A. & Cerione, R. A. *Nature* **354**, 311-314 (1991).
- Ron, D. et al. *New Biol.* **3**, 372-379 (1991).
- Adams, A. E. M., Johnson, D. I., Longnecker, R. M., Sloat, B. F. & Pringle, J. R. *J. Cell Biol.* **111**, 131-142 (1990).
- Munemitsu, S. et al. *Molec. cell Biol.* **10**, 5977-5982 (1990).
- Shinjo, K. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 9853-9857 (1990).
- Summers, M. D. & Smith, G. E. *Texas Exp. Station Bulletin N.* 1555 (1987).

- Bischoff, F. R. & Ponstingl, H. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10830-10834 (1991).
- Drivas, G. T., Shih, A., Coutavas, E., Rush, M. G. & D'Eustachio, P. *Molec. cell Biol.* **10**, 1793-1798 (1990).
- Miyamoto, S., Ohya, Y., Ohsumi, Y. & Anraku, Y. *Gene* **54**, 125-132 (1987).
- Maru, Y. & Witte, O. N. *Cell* **67**, 459-468 (1991).
- Pendergast, A. M., Muller, A. J., Havlik, M. H., Maru, Y. & Witte, O. N. *Cell* **66**, 161-171 (1991).
- Adams, J. M., Houston, H., Allen, J., Lints, T. & Harvey, R. *Oncogene* **7**, 611-618 (1982).
- Shou, C., Farnsworth, C. L., Neel, B. G. & Feig, L. A. *Nature* **358**, 351-354 (1992).
- Miyamoto, S. et al. *Biochem. biophys. Res. Commun.* **181**, 604-610 (1991).
- Prickett, K. S., Amberg, D. C. & Hopp, T. P. *Biotechniques* **7**, 580-589 (1989).
- Studier, W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. *Meth. Enzym.* **185**, 60-89 (1990).
- Vincent, S., Jeanteur, P. & Fort, P. *Molec. cell Biol.* **12**, 3138-3148 (1992).
- Yeremin, P., Chardin, P., Madaule, P. & Tavittian, A. *Nucleic Acids Res.* **15**, 1869-1869 (1987).
- Smith, D. B. & Johnson, K. S. *Gene* **67**, 31-40 (1988).

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Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis

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THE transcription factor GATA-1 is a fundamental regulator of genes in haematopoietic cell lineages^{1,2} and belongs to a family of factors that bind to the consensus sequence WGATAR³⁻⁵. The GATA motif was originally identified in *cis*-regulatory regions of globin and other erythroid-specific genes^{6,7}, but the range of genes controlled by the GATA factors has since expanded^{1,7-10}. Members of the GATA transcription factor family share a conserved zinc-finger DNA-binding domain, but the expression profile of each GATA factor is distinct¹¹⁻¹³. Here we show that a testis form of murine (m)GATA-1 messenger RNA is transcribed from a promoter located 5' to the erythroid first exon, and the remaining exons (which encode the mGATA-1 protein) are used in common by both testis and erythroid transcripts. We use an anti-mGATA-1 monoclonal antibody to show that the factor expressed in erythroid cells is the same as that found in the seminiferous tubules of murine testis. The GATA-1-expressing cells in 10-week-old testis were found only in contact with the basement membrane of seminiferous tubules, suggesting that GATA-1 regulates genes during the earliest stages of spermatogenesis.

In a search for new members of the GATA factor family, we analysed RNAs isolated from tissues of a 5-week-old mouse by blot hybridization analysis using a fragment encoding the zinc-finger domain of the mGATA-1 complementary DNA¹⁴. This was the same procedure as that used in the discovery of the GATA factor family¹¹. A testis transcript hybridized to this probe, but was unexpectedly quite stable at high stringency (Fig. 1a); the size of the transcript in testis was the same as in erythroid tissues. Use of a second probe from outside the mGATA-1 zinc-finger domain confirmed that the testis transcript was highly homologous to mGATA-1 mRNA (not shown).

We examined the developmental expression of the testis transcript, when mGATA-1 was found to be highly expressed in 2-week (prepubescent) murine testis, but steadily decreased thereafter (Fig. 1b). Very low levels were detected in adult testis, which may explain why this transcript previously escaped detection¹⁴.

A cDNA library was constructed using poly(A)⁺ RNA isolated from 2-week-old mouse testes. Sequence analysis of three testis mGATA-1 cDNAs revealed that they contained overlapping 5' untranslated (UTR) sequences which were distinct from that of the erythroid mGATA-1 cDNA¹⁴ (Fig. 2a). The complete coding sequence of the testis GATA transcript was shown to be identical to that of the erythroid mGATA-1 open reading frame¹⁴. The position of sequence divergence between erythroid and testis cDNA sequences coincides with the junction of the erythroid first exon (referred to as IE) and second exon³ (Fig. 2a; arrow), suggesting that a new testis first exon (IT) exists.

The presence of a distinct exon IT in the mGATA-1 gene was demonstrated by screening a mouse genomic DNA library using a cDNA fragment corresponding to exons IT, II and III (EB

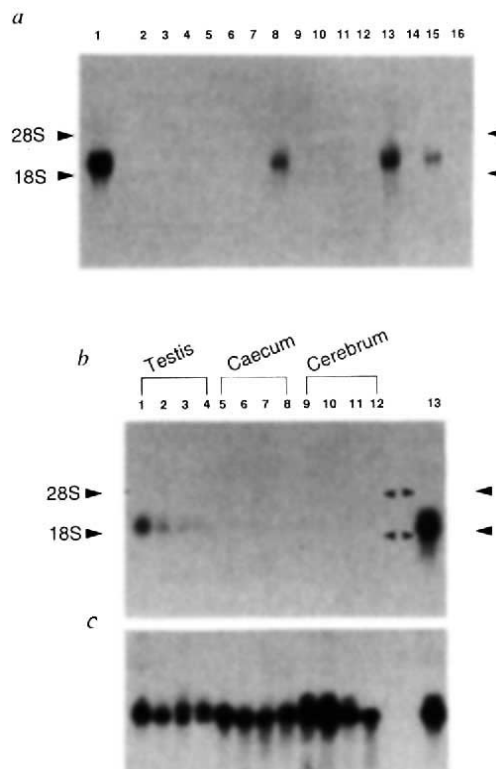


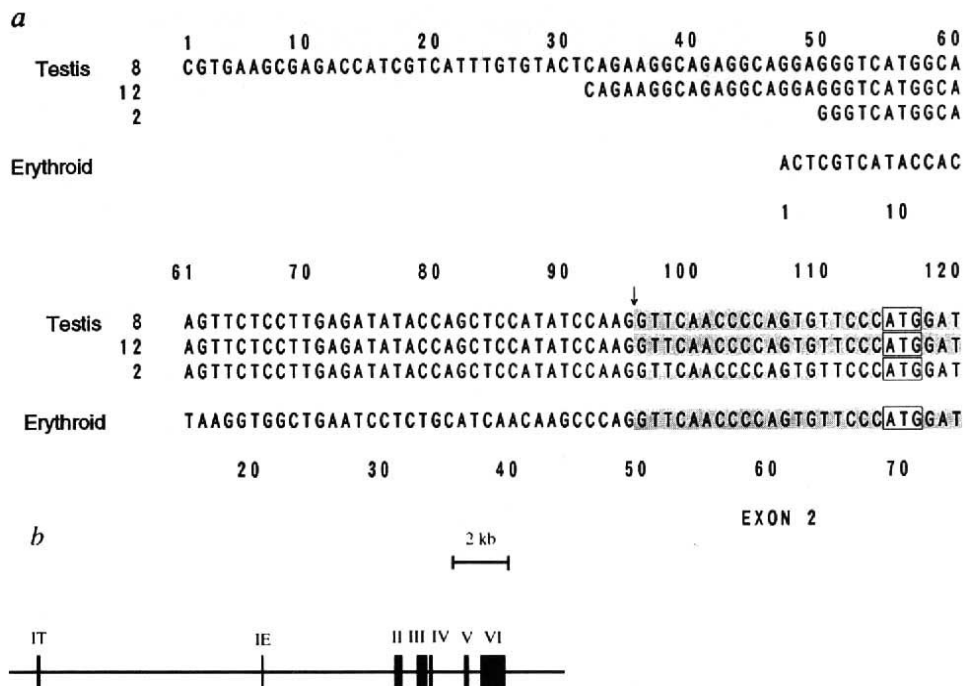
FIG. 1 Developmentally regulated expression of GATA-1 mRNA in mouse testes. *a*, Analysis of RNA from a 5-week-old mouse. RNA blot hybridization analysis¹¹ was done using 20 µg total RNA from MEL cells (lane 1), cerebrium (lane 2), cerebellum (lane 3), submandibular gland (lane 4), heart (lane 5), lung (lane 6), liver (lane 7), spleen (lane 8), lymphocyte (lane 9), muscle (lane 10), small intestine (lane 11), kidney (lane 12), bone marrow (lane 13), thymus (lane 14), testis (lane 15) and adrenal gland (lane 16). The filter was hybridized to a probe containing the zinc-finger region of mouse GATA-1 cDNA. Ribosomal RNA (18S and 28S) size migration markers are indicated. *b, c*, Analysis of RNA from mouse testes. RNA blot hybridization was done using 20 µg total RNA isolated from testes (lanes 1-4), caecum (lanes 5-8) and cerebrium (lanes 9-12) of 2-week-old (lanes 1, 5 and 9), 3-week-old (lanes 2, 6 and 10), 4-week-old (lanes 3, 7 and 11) or 5-week-old (lanes 4, 8 and 12) mice. RNA isolated from MEL cells was used as a control (lane 13). The same mGATA-1 zinc-finger probe as used for *a* was used in *b*. Ribosomal RNA (18S and 28S) markers are indicated by arrowheads. In *c*, the same filter was stripped and rehybridized to a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe to establish the amount of RNA loaded. **METHODS.** A 200-bp probe encoding the zinc-finger domain of mouse GATA-1 was synthesized by RT-PCR. Primers used in the PCR reaction were: 5'-GACAGGTCACCTACCTGTGCAA-3' (sense strand) and 5'-CACCTGATGGAGCTTGAATAGAGGCCGC-3' (antisense strand). The probe was labelled with [α -³²P]-dCTP by random priming²⁰ and used in RNA blot hybridization²¹. Formaldehyde gels for RNA electrophoresis were prepared as described¹¹.

probe; Fig. 2a)³. One genomic clone (11c) encoding exon IT, but not exons II and III, was isolated in this screen. A second genomic clone IV-2, encoding both exons IE and IT, was then isolated from another genomic library using a 3'-end fragment of clone 11c as probe. Exon IT was found to be located 8 kilobases (kb) 5' to exon IE (Fig. 2b). We inferred that the testis and erythroid forms of mGATA-1 mRNA could be transcribed from two distinct promoters (to generate alternative first exons IT or IE), which are then alternatively spliced to the coding sequence exons³.

To test this hypothesis, we used reversed transcription combined with polymerase chain reaction (RT-PCR) analysis to examine the tissue specificity of mGATA-1 expression. The results showed that mGATA-1 mRNA in the testis arises predominantly by initiation from within exon IT (Fig. 3a, lane 4),

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FIG. 2 Structure and organization of the mGATA-1 gene. **a**, Nucleotide sequence of 5'UTRs of GATA-1 cDNA. Sequence of the 5' UTR of cDNA clones (numbered 2, 8 and 12) isolated from the mouse testis cDNA library is shown with the erythroid mGATA-1 cDNA sequence¹⁴; the point at which these two cDNA sequences diverge is marked by an arrow. The translation initiation codon is boxed; the second exon common to both mRNAs is shaded. **b**, Organization of the mGATA-1 gene. The diagram is based on the results of structural analysis of genomic recombinants. Exon IE is the haematopoietic cell-specific first exon described previously³, exon IT is the testis-transcribed first exon described here. **METHODS.** A λ gt10 cDNA library (8×10^5 independent recombinants) was constructed using poly(A)⁺ RNA isolated from the testes of 2-week-old mice by standard procedures²¹. The library was screened with the zinc-finger mGATA-1 cDNA probe described in Fig. 1 legend. Positive recombinant phage were plaque-purified and subcloned into Bluescript KS(-). The complete nucleotide sequence of clone 8 and sequences of the 5' untranslated region of the other two clones were determined²². Genomic clone 11c was isolated from a strain-129 genomic DNA library (Clontech), and clone IV-2 was isolated from a new 129 library (see results) provided by T. Noda.



and that this testis transcript is contiguous with the coding sequence of mGATA-1 mRNA. In contrast, erythroid GATA-1 mRNA was found to be exclusively transcribed from exon IE in mRNA from MEL cells (not shown). We concluded that the testis and erythroid forms of mGATA-1 mRNA are mutually exclusively expressed in erythroid cells and testis.

As an initial step to determine which cell type in the testis expressed IT-initiated mGATA-1, immunoblot analysis was performed using a monoclonal antibody (N-6) raised against the bacterially expressed mGATA-1 protein. The N-6 antibody identified a single band of $M_r \sim 50,000$ (50K) in the testis and spleen of a 2-week-old mouse (Fig. 3b), as well as in MEL cell extracts (Fig. 3b, lane 3; ref. 14). As the testis and erythroid mGATA-1 cDNAs differ only in the 5'UTR, this suggests that the mGATA-1 protein expressed in both tissues is identical; consistent with this hypothesis, the monoclonal antibody N-6 recognizes an apparently identical protein in testis and MEL cells (Fig. 3b).

We then used antibody N-6 in cytological analysis to identify the specific cells in the testis that express mGATA-1. Three types of cells exist in the testis: spermatogonia (germ-line cells), Sertoli cells (which provide the appropriate microenvironment for spermatogenesis) and Leydig cells (interstitial cells between tubules)^{15,16}. In 2-week-old testis, round mGATA-1-positive cells displaying prominent nuclear staining were detected at relatively high frequency inside the seminiferous tubules; thus the mGATA-1-expressing cells in testis are not Leydig cells^{15,16} (Fig. 4a). In 5-week (not shown) or 10-week testis, mGATA-1 expression was observed in only a small number of peripheral cells in association with the basement membrane of the seminiferous tubules (Fig. 4b); curiously, the cytoplasm of the cells from the more mature animals was most prominently stained with the N-6 antibody. The ratio of GATA-1 antibody-positive cells to total cells in the seminiferous tubules was much higher in 2-week testis than in 5- or 10-week tissue (Fig. 4b), in agreement with the developmental expression profile of mGATA-1 mRNA (Fig. 1b).

As spermatocyte differentiation progresses both by radially inward movement of germ cells from the periphery of the semi-

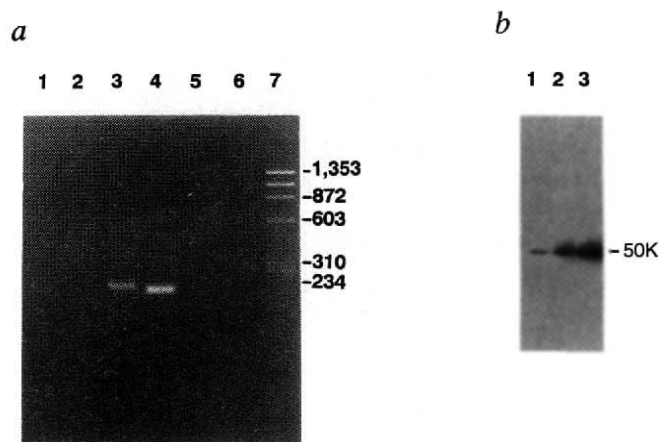


FIG. 3 Tissue specificity of GATA-1 expression. **a**, RT-PCR analysis of GATA-1 mRNA expression. RT-PCR was performed on mRNA isolated from testis (lanes 1, 4) or brain (lanes 2, 5) of a 2-week-old mouse or MEL cells (lanes 3, 6) using primers specific for the zinc-finger region (lanes 1-3) or the testis form of mGATA-1 (lanes 4-6). Numbers on the right are size in nucleotides. **b**, Western blot analysis. GATA-1 factor expression in the testis (lane 1) and spleen (lane 2) of a 2-week-old mouse and in MEL cells (lane 3) was assayed by immunoblot analysis using the N-6 anti-GATA-1 monoclonal antibody.

METHODS. Total RNA (1 μ g) from the testes or brain of a 2-week-old mouse, and from MEL cells, was reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (BRL) and random primers (Pharmacia). The cDNA was used as template for PCR amplification by *Taq* DNA polymerase (Takara Shuzo). To examine the tissue specificity of the testis transcript, the reaction mixture was amplified using a set of primers (5'-CCGAATTCGGTGAAGCGAGACCATCGTC-3' (sense) and 5'-CCGAATTCAGGGCAGAAATCCACAAAC-3' (antisense)) corresponding to exon IT and exon II, respectively (with *EcoRI* linkers added to the ends). The N-6 monoclonal antibody was prepared using mGATA-1 protein expressed in *Escherichia coli* as antigen. The cloned myeloma line producing monoclonal N-6 was identified as a rat anti-mouse GATA-1 antibody by supershift of GATA-1:GATA site complex in electrophoretic gel mobility shift assay (L.G., M.Y. and J.D.E., unpublished observations). The testis or spleen from a 2-week-old mouse was homogenized directly in SDS-gel sample buffer and proteins were then separated by SDS-PAGE²³. After transfer to a PVDF membrane, the membrane was reacted with the N-6 antibody (1:100 dilution of the ascites solution). Specific binding was visualized using the horseradish peroxidase-conjugated second antibody ECL system (Amersham).

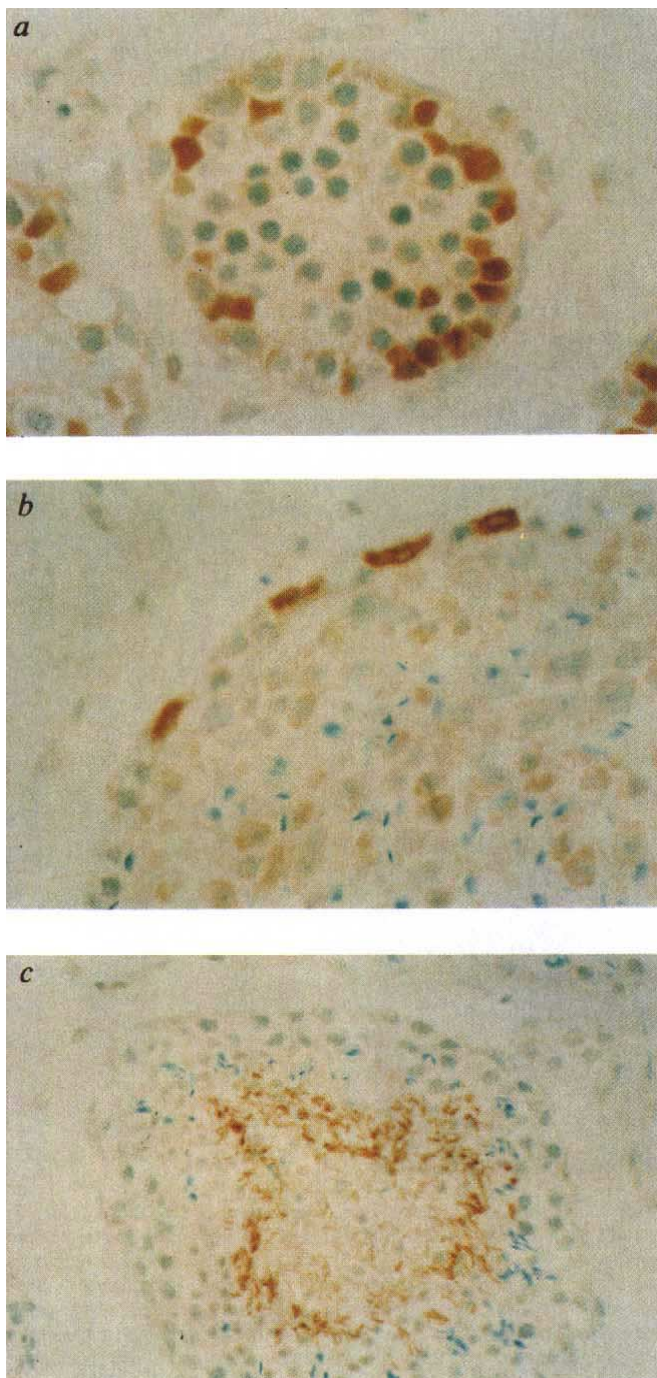


FIG. 4 Cytological analysis of GATA-1 expression in testis. Thin sections from 2-week-old (a) or 10-week-old (b) murine testes were stained using the N-6 antibody; c, a thin section of the 10-week-old mouse testis was also stained with a rat monoclonal antibody against human procollagen I amino-terminus (Chemicon, CA) which was found to be crossreactive with mouse sperm.

METHODS. Sliced mouse testes at 2, 5 or 10 weeks old were fixed in periodate-lysine-4% paraformaldehyde for 2 h. Testes were washed in PBS with 20% sucrose and were rapidly frozen after embedding in OCT compound (Miles). 3-mm-thick frozen sections were processed for immunohistochemistry. After rinsing in sheep serum, sections were reacted with N-6 antibody (1:5 to 1:20 dilution of ascites) for 24 h at 4 °C. After washing, the specimens were incubated with horseradish peroxidase-conjugated F(ab)₂ fragment of anti-rat IgG (Amersham; 1:200 dilution) overnight at 4 °C; diaminobenzidine was used as chromogen. Normal mouse serum was added to the second antibody solution to block nonspecific reaction. In control experiments, no primary antibody was added.

niferous tubules towards the central lumen and by lateral maturation along the tubule, each tubule cross-section represents a series of concentric rings of germ cells at the same stage of differentiation^{15,16}. The GATA-1-staining cells were at the periphery of the tubules (Fig. 4a, b) not associated with mature sperm (Fig. 4c), and thus the immunohistochemical evidence suggests that GATA-1 is found only in cells at a very early stage of spermatogenesis. Alternatively, these could represent a stage of Sertoli cells or testis cells which have not yet been described.

We previously reported the identification of the GATA-2 and -3 factors, which demonstrated that the GATA motif directs a more complicated array of developmental regulatory processes than those that function solely in cells of the erythroid/myeloid lineage¹¹. Recent data strongly suggest the existence of an additional tissue-restricted transcription factor (OSP-1) with properties expected for a GATA protein¹⁷. OSP-1 binds to a GATA consensus sequence within the murine zona pellucida-3 gene promoter and that binding site is functionally required for appropriate expression of the gene. The novel mGATA-1 transcription initiation site reported here may therefore not be testis-restricted. Our initial experiments, however, have failed to demonstrate that this alternative promoter is used in the female germ line. The present study implicates GATA-1 as an important effector in spermatogenesis. The developmentally regulated modulation of GATA-1 in the testis may reflect the significance of this protein in germ-cell generation, perhaps equivalent to the pivotal role the same protein plays in haematopoiesis.

In embryonic stem (ES) cells in which the single copy of mGATA-1 (GATA-1 is on the X chromosome) was ablated by homologous recombination, the most severely affected developmental process due to the mutation is in red blood cells¹⁸. From our results it might be anticipated that mGATA-1⁻-ES-reconstituted mice may also fail to contribute to the male germ line. An interesting possibility is that in addition to its demonstrated role in erythroid cell development¹⁹ and its anticipated role in germ-cell generation proposed here, the GATA-1 factor transcribed from the IT promoter may contribute to the developmental programme very early in haematopoiesis. Direct tests of the possibilities discussed here by investigating the physiological defect(s) resulting from deletion of the IT exon from the mouse germ line are underway. □

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- Orkin, S. H. *Blood* **80**, 575-581 (1992).
- Yang, Z. et al. in *The Regulation of Hemoglobin Switching* (eds Stamatoyannopoulos, G. & Nienhuis, A.) 249-265 (Johns Hopkins Univ. Press, Baltimore, MD, 1991).
- Tsai, S. F., Strauss, E. & Orkin, S. H. *Genes Dev.* **5**, 919-931 (1991).
- Wall, L., DeBoer, E. & Grosfeld, F. *Genes Dev.* **2**, 1089-1100 (1989).
- Plump, M. et al. *Nucleic Acids Res.* **17**, 73-92 (1989).
- Evans, T., Reitman, M. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5976-5980 (1988).
- Mignotte, V., Eleouet, J. F., Raich, N. & Romeo, P.-H. *Proc. natn. Acad. Sci. U.S.A.* **17**, 37-54 (1989).
- Ko, L. J. et al. *Molec. cell. Biol.* **11**, 2778-2784 (1991).
- Redondo, J. M., Pfohl, J. L. & Krangel, M. S. *Molec. cell. Biol.* **11**, 5671-5680 (1991).
- Wilson, D. B., Dorfman, D. M. & Orkin, S. H. *Molec. cell. Biol.* **10**, 4854-4862 (1990).
- Yamamoto, M. et al. *Genes Dev.* **4**, 1650-1662 (1990).
- Martin, D. I. K., Zon, L. I., Mutter, G. & Orkins, S. H. *Nature* **344**, 444-447 (1990).
- Romeo, P.-H. et al. *Nature* **344**, 447-449 (1990).
- Tsai, S. F. et al. *Nature* **339**, 446-451 (1989).
- Willison, K. & Ashworth, A. *Trends Genet.* **3**, 351-355 (1987).
- Fawcett, D. W. in *A Textbook of Histology* 11th edn, 796-848 (Saunders, Philadelphia, 1986).
- Schickler, M., Lira, S. A., Kinloch, R. A. & Wasserman, P. M. *Molec. cell. Biol.* **12**, 120-127 (1992).
- Pevny, L. et al. *Nature* **349**, 257-260 (1991).
- Simon, M. C. et al. *Nature Genet.* **1**, 92-98 (1992).
- Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6-13 (1983).
- Sambrook, J., Fritsch, E. F. & Maniatis, T. in *Molecular Cloning: A Laboratory Manual* 2nd edn (Cold Spring Harbor Press, New York, 1989).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).

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Three-dimensional cryo-electron microscopy of the calcium ion pump in the sarcoplasmic reticulum membrane

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THE ATP-driven calcium pump (Ca^{2+} -ATPase) is an integral membrane protein (M_r 110K) which relaxes striated muscle by pumping calcium out of the cytoplasm into the sarcoplasmic reticulum against a large concentration gradient¹. Recent efforts have attempted to relate the sequence of Ca^{2+} -ATPase to its structure and function. In particular, site-directed mutagenesis has identified critical amino-acid residues²⁻⁶, and its predicted secondary structure, which includes ten transmembrane helices⁷, has gained experimental support⁸⁻¹⁰. But direct visualization of the molecule has so far been limited to the cytoplasmic domains at low resolution^{11,12}. We present here the three-dimensional structure of Ca^{2+} -ATPase in the native sarcoplasmic reticulum membrane at 14 Å resolution, determined by cryo-electron microscopy and helical image analysis. The structure shows an unexpected transmembrane organization, consisting of three distinct segments, one of which is highly inclined. These features can be related to earlier predictions of secondary structure.

Tubular crystals of Ca^{2+} -ATPase in native membranes were grown by incubating sarcoplasmic reticulum (SR) in EGTA and vanadate¹³. Images of these tubes embedded in amorphous ice (Fig. 1a) diffracted to 14 Å resolution (Fig. 1b), and three-dimensional structures were constructed using helical symmetry¹⁴. Thus, we determined three independent structures with different helical symmetries, each from averaged data sets (see Fig. 1c, for example). As the molecular features are the same, we will describe only one of these structures in detail. A surface rendering (Fig. 2) shows the arrangement of molecules in the SR membrane. Consistent with previous reconstructions^{11,12}, two strands of Ca^{2+} -ATPase molecules pointing in opposite directions (Fig. 2a, arrowheads) follow a helical track to form a 'dimer ribbon'. Each molecule (Fig. 2a, dotted area) appears to be linked to the twofold-related one on the opposite strand by a bridge between the tops of the molecules. The dimer ribbons are further stabilized by diagonal connections between the strands (Fig. 2, black stars); contacts between the ribbons are made by the small luminal domains (Fig. 2b, white asterisks, arrowed). As expected, the bilayer structure of the membrane (M) appears here as two nearly continuous bands (Fig. 2b). Because the contrast is minimal at the outer surfaces of these bands (horizontal bars in Fig. 2b), we presume that the phospholipid head groups are located there. The thickness of the membrane thus defined is unexpectedly thin, with only 32 Å between the horizontal bars.

Ca^{2+} -ATPase is a tall molecule (Fig. 3a), consisting of cytoplasmic, transmembrane and luminal parts. The molecule fits into a box of 120 Å (height) $\times$ 50 Å (perpendicular to the dimer ribbons) $\times$ 85 Å (along the dimer ribbons). Consistent with previous observations^{11,12,15,16}, it has a highly asymmetric mass distribution across the lipid bilayer: the cytoplasmic part comprises ~70% of the total mass, whereas the luminal part occupies

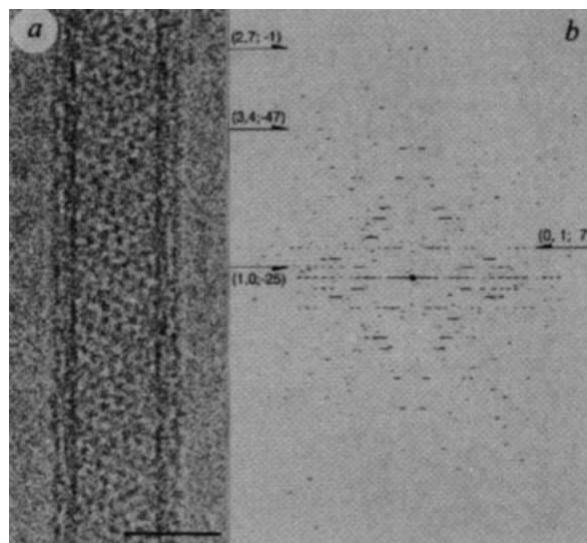


FIG. 1 Electron micrograph of a tubular crystal of Ca^{2+} -ATPase embedded in amorphous ice (a) together with its Fourier transform (b). The micrograph shows only ~1/4 of the overall length used for image reconstruction. The transform consists of a series of horizontal lines (layer lines), each corresponding to a set of helices. Each set of helices has been characterized by an (h,k) index and its start number (n) on an associated surface lattice¹⁴. A clear, near-meridional layer line ($h=2, k=7; n=-1$) at $1/16 \text{ Å}^{-1}$ was often seen (b). After averaging data sets from 4 tubes, phases were consistent with twofold symmetry to 14 Å resolution. The amplitudes and phases along a major $(0,1;7)$ and a high-order $(3,4;-47)$ layer line are shown in c. Phases show a clear tendency to be either 0° or 180° , indicative of twofold symmetry, especially where the amplitudes are strong. Bar in a represents 500 Å.

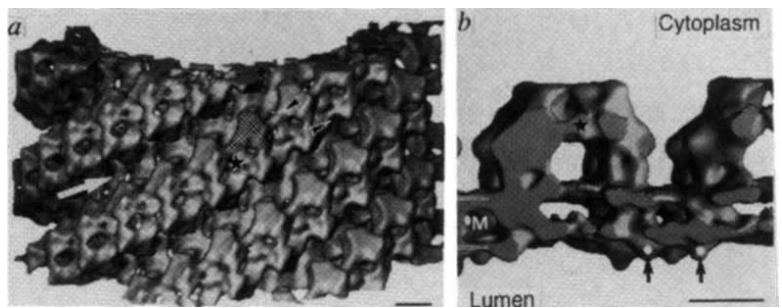
METHODS. SR was prepared according to ref. 21. Crystals were induced by incubating SR overnight at 0°C in a buffer containing 100 mM KCl, 5 mM MgCl_2 , 10 mM imidazole, 0.5 mM EGTA, 5 mM Na_3VO_4 , pH adjusted to 7.3 (ref. 13). The specimen was put on a holey carbon film and rapidly frozen by plunging into an ethane slush²². Gatan cryoholders were used for examination in a Philips EM420T or JEOL JEM2000EX microscope at a magnification of $36,000\times$ (EM420T at 120 kV); the nominal magnification was $40,000\times$ for JEM2000EX operated at 200 kV. Suitable images were digitized at 20- μm intervals and used for helical reconstruction as described¹⁴. Altogether, 47 layer lines were used for the final reconstruction after enforcing twofold symmetry; equatorial data were included at full weight. The amplitude-weighted phase residual for twofold symmetry was 10.5° , including all the Fourier terms (except for the equatorial data) with amplitudes higher than 2% of the highest off-equatorial peak.

only ~5%. The cytoplasmic part has a complex structure consisting of several domains, and resembles the head and neck of a bird. The 'head' (H in Fig. 3a, b) is responsible for forming dimer ribbons and contains domains for ATP binding and phosphorylation. The 'stalk' (S), connecting the head to the transmembrane domain, is ~25 Å long and consists of two segments. At the top of the stalk, these segments separate to form a cavity, which can be seen by looking along the dimer ribbons, that is, at the back of the head (Fig. 3c, d).

The transmembrane part (M) consists of three segments (marked A, B, and C in Figs 3 and 4), which are clearly resolved in the hydrophobic core of the membrane (Fig. 3e). The largest segment (A) consists of two parts, one vertical (A2) and the other inclined (A1) so that the two parts separate as the luminal surface is approached (Fig. 3c). At this point, A1 ends abruptly, but on the cytoplasmic side it continues into the stalk. In contrast, A2 is displaced from the stalk (Fig. 3c) but is connected to the highly inclined B segment at both surfaces of the membrane (Fig. 3a). Owing to its inclination (~40°; Fig. 3a), B merges with A2 on the cytoplasmic side, but at the luminal surface B is ~40 Å away from A2, to which it is relinked through the small luminal domain (white asterisk in Fig. 3a, c, d). The

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FIG. 2 Surface models of portions of the tubular crystal of Ca^{2+} -ATPase. *a*, Front half of the tube showing the cytoplasmic side; *b*, view along the membrane surface in the direction specified by the white arrow in *a*. Note in *b* that the two leaflets of the lipid bilayer (M) are clearly resolved and slightly curved; the curvature is due to the cylindrical nature of these tubes. Most of the protein protrudes into the cytoplasm, where molecules are linked together into 'dimer ribbons'. The dotted area in *a* represents one molecule that apparently forms a dimer with the molecule below. Of several connections between molecules, the diagonal ones, which are mediated by a high-density peak at the twofold axis (black star), seem to be most effective in linking the two strands of the dimer ribbon (black arrowheads in *a*). The small domains on the luminal surface (white asterisks (arrowed) in *b*) appear to make a link between dimer ribbons. The density cutoff level for the model was chosen so that $\sim 100\%$ of the expected volume was recovered (assuming an M_r of 110K, and a partial specific volume of $0.74 \text{ cm}^3 \text{ g}^{-1}$ based on



the amino-acid composition). At this cutoff level, the membrane surface is not completely continuous. Bars represent $50 \text{ \AA}$.

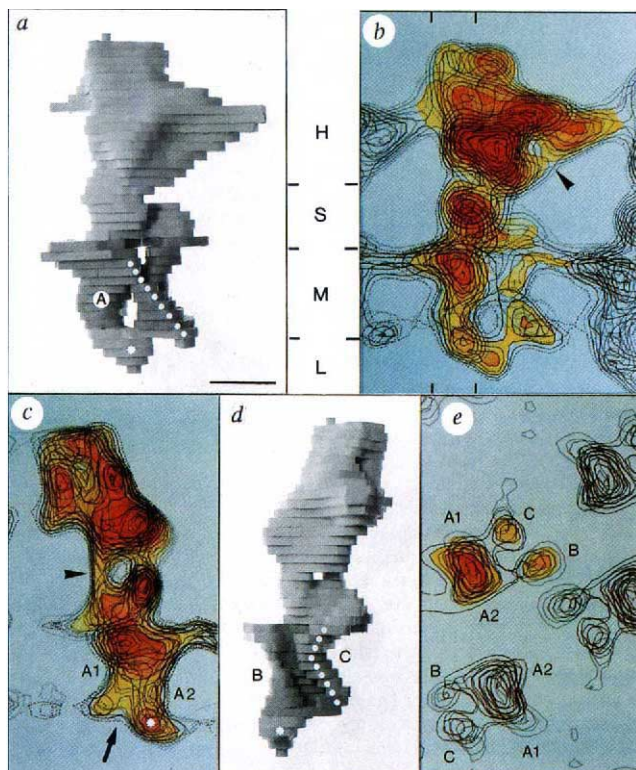


FIG. 3 Three-dimensional structure of the Ca^{2+} -ATPase molecule in the lipid bilayer. Views perpendicular to the dimer ribbon are shown in *a* and *b*, along the ribbon in *c* and *d*, and normal to the membrane in *e*. Equivalent views of a wooden model (*a*) and a stack of transparent sections (*b*) show the domains constituting the molecule: H, head portion of the cytoplasmic domain; S, stalk; M, membrane; L, luminal domain. Two nearly continuous densities flanking M represent two leaflets of the lipid bilayer. The white dotted line, inclined $\sim 40^\circ$ from vertical, approximates the path of segment B. *c*, A stack of sections ($19 \text{ \AA}$ thick) showing the structure of A segment and the stalk. The sections are cut normal to the 'dimer ribbon' and cover the area specified in *b* by vertical bars at the top and bottom margins. Note that the two segments of A (A1 and A2) are well separated near the luminal surface of the membrane (arrow). The arrowhead indicates the cavity at the top of the stalk region. *d*, A view of the wooden model in *a* looking from right to left; white dots approximate the path of segment C. *e*, Stack of sections ($13 \text{ \AA}$ thick) showing the three transmembrane segments. Sections are cut parallel to the membrane surface and represent the structure at the middle of the bilayer (hydrophobic core). The volume of the solid model, cut out at the lowest solid contour in *b*, *c* and *e*, is $99,000 \text{ \AA}^3$, corresponding to $\sim 75\%$ of the expected volume. The transparent sections have been shaded to emphasize high-density regions of a selected molecule (*b*, *c*, *e*). Scale bar corresponds to $25 \text{ \AA}$.

third membrane segment (C) is curved and appears to extend $\sim 20 \text{ \AA}$ into the cytoplasm (Fig. 3*d*); this $\sim 60 \text{ \AA}$ -long cylinder may represent a single, continuous piece of secondary structure. The cross-section of C is almost constant throughout the bilayer, unlike that of B, which becomes larger towards the luminal surface.

Gross features of the reconstructed structure, such as the distribution of mass among the various domains and the segmentation of the stalk, are consistent with the '10-helix model'¹⁷, which is shown in Fig. 4*b*. In addition, several detailed features fit the predictions of this model (summarized in Fig. 4*a*). Specifically, it predicts that the seventh transmembrane helix (M7) is particularly long and could therefore accommodate an inclination of $\sim 40^\circ$ from vertical. Furthermore, the loop between M7 and M8 constitutes almost half the mass on the luminal side (32 of 72 predicted residues) and has been shown to be antigenic⁹, suggesting that it is well exposed. Thus, we propose that transmembrane segment B contains M7 (possibly together with another helix) and that the small luminal mass contains the M7-M8 loop. Accordingly, M8 must run through transmembrane segment A2. Assignment of segment C is more difficult because, although this curved segment should also contain a somewhat longer hydrophobic sequence, none of the remaining putative transmembrane helices has a particularly long sequence. One plausible interpretation based on the 10-helix model is to place M1 within segment C. According to the original model, the stalk consists of five helices (denoted as S1-S5 in Fig. 4*b*), which are contiguous with transmembrane helices M1-M5. But if we consider the related sequence of Na^+/K^+ -ATPase, S1 could be plausibly excluded because of a distinctly non-helical sequence (...PPPTTP...) near the middle¹⁸. Furthermore, the length of segment C together with its cytoplasmic extension is $\sim 60 \text{ \AA}$, and would thus accommodate the sequence of M1 plus S1 (40 residues).

Where is the transmembrane ion pathway located? Four transmembrane helices (M4, M5, M6 and M8) have been implicated by site-directed mutagenesis in Ca^{2+} binding¹⁹. We propose that these helices cluster at the interface of transmembrane segments A1 and A2 in our reconstruction. According to the 10-helix model (Fig. 4*b*), M4 and M5 are connected to stalk helices (S4 and S5) and are therefore likely to be located in the A1 segment. M8 is connected to M7 by the luminal domain and hence assigned to the A2 segment. This leaves M6, which is predicted to be displaced from the stalk (Fig. 4*b*) and is therefore likely to be located in A2 segment. It is interesting to note that, because A1 is slanted and A2 is vertical, there is an outlet at the luminal surface between segments A1 and A2 (arrow in Fig. 3*c*); the Ca^{2+} sites are expected to face the lumen in the conformation (termed E_2) that has been proposed to form these tubular crystals¹³.

The map may also give a clue to the location of the ATP binding site. There are two grooves or cavities in the cytoplasmic

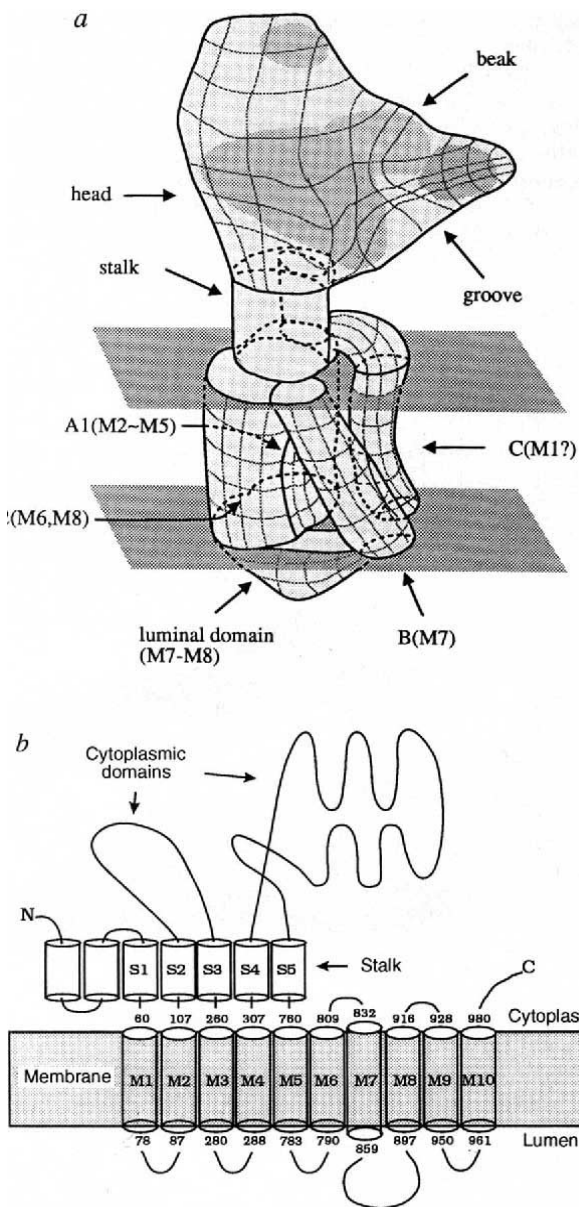


FIG. 4 Relationship between the three-dimensional structure of Ca^{2+} -ATPase and earlier predictions of secondary structure based on amino-acid sequence (the 10-helix model¹⁷). *a*, Structure of the Ca^{2+} -ATPase molecule. The transmembrane domain has 3 segments (A, B and C), and the luminal domain connects segments A and B. The furthest part of segment A (A1) extends up into the cytoplasmic stalk, whereas the closer part (A2) merges at the cytoplasmic side of the membrane with segment B, and at the luminal side with the small extramembranous domain. Assignments for 8 out of 10 putative transmembrane helices, as predicted by the 10-helix model, are indicated in parentheses. *b*, Diagram showing the predicted secondary structure with 10 transmembrane helices (M1-M10) and 5 stalk helices (S1-S5) (adapted from ref. 17). The numbers above and below the membrane indicate positions of the residues at each end of the transmembrane helices.

part, one near the base of the 'beak' (arrowhead in Fig. 3b), and the other at the top of the stalk region (arrowhead in Fig. 3c). If we speculate that one of these cavities represents the ATP site, then the one near the base of the beak is more plausible on the basis of results from affinity labelling. These indicate that ATP binds at the intersection of three cytoplasmic domains (reviewed in ref. 20), and three domains do indeed appear to surround this groove (Fig. 3b). The cavity in the stalk might play a part in transmitting conformation changes caused by phosphorylation to the transmembrane region. □

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1. Inesi, G., Lewis, D., Nikic, D., Hussain, A. & Kirtley, M. E. *Adv. Enzym.* **65**, 185-215 (1992).
2. Clarke, D. M., Loo, T. W. & MacLennan, D. H. *J. biol. Chem.* **265**, 22223-22227 (1990).
3. Clarke, D. M., Loo, T. W. & MacLennan, D. H. *J. biol. Chem.* **265**, 6262-6267 (1990).
4. Clarke, D. M. *et al.* *J. biol. Chem.* **264**, 11246-11251 (1989).
5. Vilsen, B., Andersen, J. P., Clarke, D. M. & MacLennan, D. H. *J. biol. Chem.* **264**, 21024-21030 (1989).
6. Andersen, J. P., Vilsen, B., Leberer, E. & MacLennan, D. H. *J. biol. Chem.* **264**, 21018-21023 (1989).
7. MacLennan, D. H., Brandl, C. J., Korczak, B. & Green, N. M. *Nature* **316**, 696-700 (1985).
8. Matthews, I., Sharma, R. P., Lee, A. G. & East, J. M. *J. biol. Chem.* **265**, 18737-18740 (1990).
9. Clarke, D. M., Loo, T. W. & MacLennan, D. H. *J. biol. Chem.* **265**, 17405-17408 (1990).
10. Sachs, G. *et al.* *J. Bioenerg. Biomembr.* **24**, 301-308 (1992).
11. Taylor, K. A., Dux, L. & Martonosi, A. *J. molec. Biol.* **187**, 417-427 (1986).
12. Castellani, L., Hardwicke, P. M. & Vibert, P. *J. molec. Biol.* **185**, 579-594 (1985).
13. Dux, L. & Martonosi, A. *J. biol. Chem.* **258**, 2599-2603 (1983).
14. Toyoshima, C. & Unwin, N. *J. Cell Biol.* **111**, 2623-2635 (1990).
15. Herbetle, L. *et al.* *Biochim. biophys. Acta* **817**, 103-122 (1985).
16. Dupont, Y., Harrison, S. C. & Hasselbach, W. *Nature* **244**, 555-558 (1973).
17. Brandl, C. J., Green, N. M., Korczak, B. & MacLennan, D. H. *Cell* **44**, 597-607 (1986).
18. Green, N. M. *Biochem. Soc. Trans.* **17**, 970-972 (1989).
19. Clarke, D. M., Loo, T. W., Inesi, G. & MacLennan, D. H. *Nature* **339**, 476-478 (1989).
20. Green, N. M. & Stokes, D. L. *Acta Physiol. scand.* **146**, 59-68 (1992).
21. Meissner, G., Conner, G. E. & Fleischer, S. *Biochim. biophys. Acta* **298**, 246-269 (1973).
22. Toyoshima, C. *Ultramicroscopy* **30**, 439-444 (1989).

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Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*

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EDYSONE in *Drosophila* has been a paradigm for steroid hormones since its ability to induce gene activity directly was demonstrated by its effects on moulting and polytene chromosome puffing¹⁻³. The ecdysone receptor (EcR) was recently confirmed as a member of the nuclear receptor superfamily by cloning and characterization in a *Drosophila* cell line⁴. Here we show that EcR needs to heterodimerize with either the retinoid X receptor (RXR)⁵ or its *Drosophila* homologue, *ultraspiracle* (USP)⁶, for DNA binding and transactivation. These results place the ecdysone receptor in the heterodimerizing class of the nuclear receptor superfamily and demonstrate that the role of RXR/USP as a central and promiscuous partner in mediating the activity of these receptors⁷⁻¹² is highly conserved. Whereas EcR-USP DNA-binding activity is unaffected by hormone, EcR-RXR DNA-binding activity is stimulated by either ecdysteroid or 9-*cis*-retinoic acid, demonstrating that hormone can play a role in heterodimer stabilization.

Using transient cotransfection assays in mammalian cell lines, we found that HeLa cells could support ecdysone-responsive transactivation (data not shown), whereas other mammalian cell lines, in particular CV1, did not (see later). To investigate this difference, EcR was transfected into both HeLa and CV1 cell lines, and extracts prepared. Gel shift experiments showed that a complex that was readily detectable in the transfected HeLa extract was only weakly present in the transfected CV1 extract (Fig. 1a, lanes 4 and 5), indicating that the difference between the two cell lines was reflected as differences of EcR-dependent DNA-binding activity. Consequently we examined the DNA-binding properties of EcR translated *in vitro*. This EcR by itself did not detectably bind oligonucleotides containing an ecdysone response element (Ec-RE)^{13,14}, but a complex was produced when HeLa extract was added (Fig. 1b, lanes 1 and 5). Adding

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CV1 extract also produced a complex with the same mobility, albeit in much smaller quantities than with HeLa extracts (data not shown). When a truncated EcR was mixed with HeLa extract, a faster migrating complex was observed (Fig. 1b, lanes 2 and 6). When both the complete and truncated EcRs were mixed or cotranslated and then mixed with HeLa extract, no complex with intermediate mobility appeared, suggesting that EcR does not associate with itself in the complex that binds the Ec-RE (Fig. 1b, lanes 3, 4, 7–12).

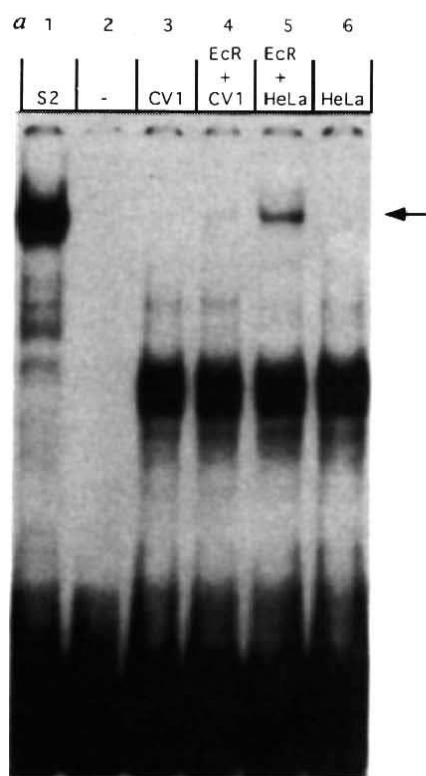
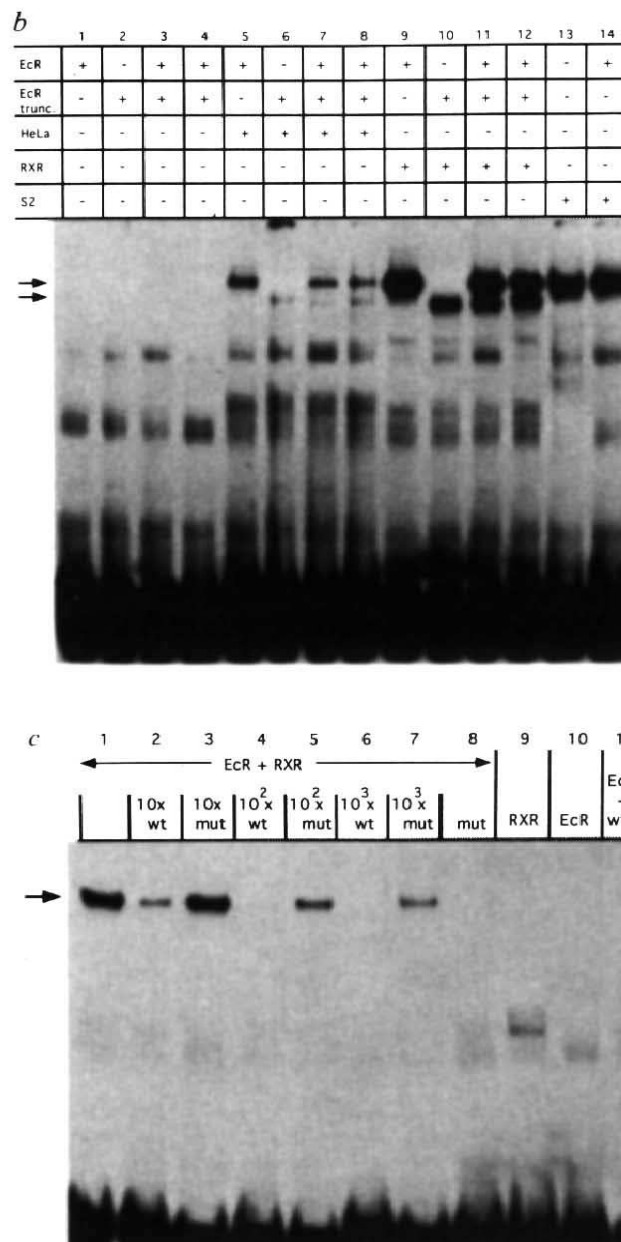


FIG. 1 The ecdysone receptor (EcR) binds DNA as a heterodimer in gel shift experiments. Arrows indicate the positions of specific complexes. *a*, Extracts prepared from S2, CV1 or HeLa cells transfected or not with cytomegalovirus (CMV)-EcR, were mixed with wild-type (wt) ecdysone response element (Ec-RE) oligonucleotide. Lane 1, *Drosophila* S2 extract; lane 2, no added protein; lane 3, CV1 cell extract; lane 4, extract of CV1 cells transfected with CMV-EcR; lane 5, extract of HeLa cells transfected with CMV-EcR; lane 6, HeLa cell extract. *b*, *In vitro*-translated EcR, either full-length or truncated (trunc.), as indicated, were mixed (lanes 3, 7, 11) or cotranslated (lanes 4, 8, 12) and then mixed with HeLa extract (lanes 5–8), vaccinia extract containing RXR- α (lanes 9–12), or S2 extract (lanes 13–15). Lane 16, no added protein. Wild-type Ec-RE oligonucleotide was then added. *c*, EcR and RXR- α were mixed and bound to wt Ec-RE oligonucleotide in the presence of excess unlabelled wt Ec-RE or of excess mutated Ec-RE oligonucleotides as indicated (lanes 1–7). Lane 8, EcR plus RXR- α incubated with radioactively labelled mutated Ec-RE oligonucleotide. Results similar to those shown in lanes 1–8 were obtained with the S2 activity and the EcR HeLa extracts (data not shown). Lanes 9–12, wt Ec-RE oligonucleotide plus RXR- α -vaccinia extract only, EcR only, EcR plus wt vaccinia extract, wt vaccinia extract only, respectively.

METHODS. Gel shift experiments were done with blunt-ended double-stranded oligonucleotides, using wild-type Ec-RE CTAGAAGGTTCAATGCACT-TAGATC, mutated Ec-RE CTAGAATGCACAACTGCACTTAGATC, or the retinoic acid response element RA-RE- β 2 oligonucleotide¹¹. HeLa or CV1 extracts were prepared according to ref. 23, except that the extraction buffer contained 150 mM NaCl; 10 μ l (*a*) or 4 μ l (*b* and *c*) of extract was used. In *a*, equal amounts of CV1 and HeLa extracts were tested by determining the amount of gel shift activity produced on an Oct-1 oligonucleotide by transfected CMV-Oct 4 and by the endogenous Oct-binding proteins²³ (data not shown). CMV-EcR was constructed by cloning the *Fsp*I/*Hind*III fragment of the EcR cDNA⁴ into *Not*I-cut CMV- β ²⁴. Almost confluent 10-cm dishes of CV1 or HeLa cells were transfected with 10 μ g CMV-EcR mixed with 140 μ l DOTAP (Boehringer Mannheim) according to the manufacturer's instructions

As the Ec-RE contains half-sites similar to those recognized by members of other nuclear receptor superfamilies^{4,14}, we mixed EcR with several possible heterodimer partners. DNA binding was observed when EcR was mixed with RXR- α (ref. 5) (Fig. 1b, lanes 1 and 9; Fig. 1c, lanes 1, 9 and 10) but not with the retinoic acid α -receptor^{15,16} (RAR- α), thyroid hormone α -receptor¹⁷ or COUP-TF (ref. 18; data not shown). Moreover, truncated EcR when mixed with S2 extract competed for the endogenous S2 complex, producing a faster migrating species



and collected after 24 h. For *in vitro* translation, we used 35 μ l rabbit reticulate lysate (Promega) plus 0.2 μ g T7 or T3 RNA polymerase transcripts from BS-EcR, BS-EcR short or pSK USP in a total reaction volume of 50 μ l. After protein synthesis, 2 μ l was taken for each gel shift reaction. Vaccinia extracts were prepared according to ref. 11; 0.1 μ l was assayed. S2 extract was prepared as described²⁵ (gift from R. Paro). *In vitro*-translated proteins and extracts were mixed or not, as indicated, and incubated on ice for 15 min in the presence of 10^{-6} M muristerone, whereupon oligonucleotides were added and incubated for a further 15 min on ice. Electrophoresis was done as described¹¹. All templates for *in vitro* transcription used pBluescript plasmids; construction details for all plasmids are available on request. Truncated EcR was created by removing sequences 3' to the *Nar*I site at nucleotide 3,121 of EcR cDNA⁴ and introducing stop codons.

FIG. 2 EcR-USP and RAR- α -USP heterodimerization detected by gel shift analysis. Lanes 1-4, wild-type (wt) Ec-RE oligonucleotide plus S2 extract, *in vitro*-translated USP, *in vitro*-translated EcR, alone or mixed, as indicated. Lanes 5-8, RA-RE- β 2 oligonucleotide plus RAR- α -vaccinia extract mixed with RXR- α -vaccinia extract, *in vitro*-translated USP or RAR- α -vaccinia extract, alone or mixed as indicated. Arrow indicates the specific complex; other complexes are derived from reticulate lysate or extract backgrounds and are nonspecific (Fig. 1c, and data not shown). For methods, see Fig. 1 legend.

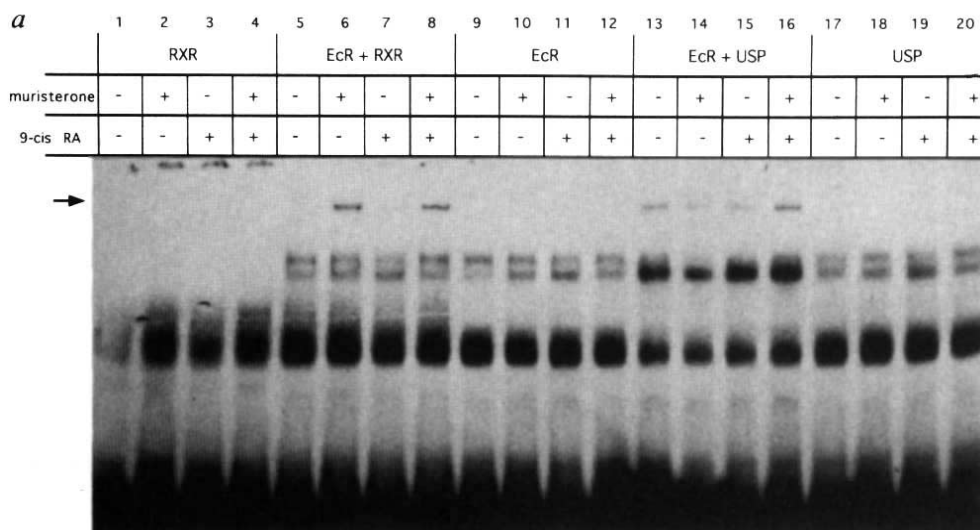
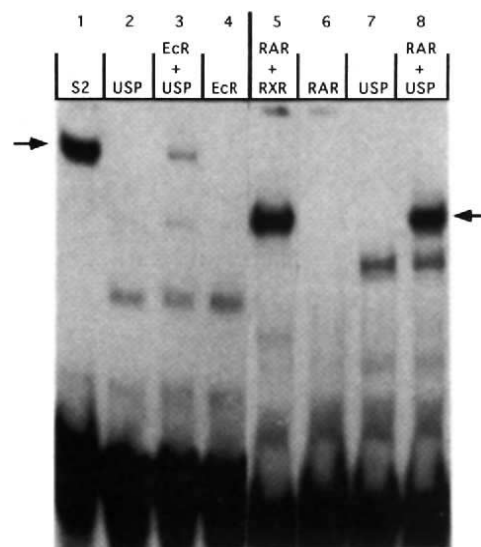
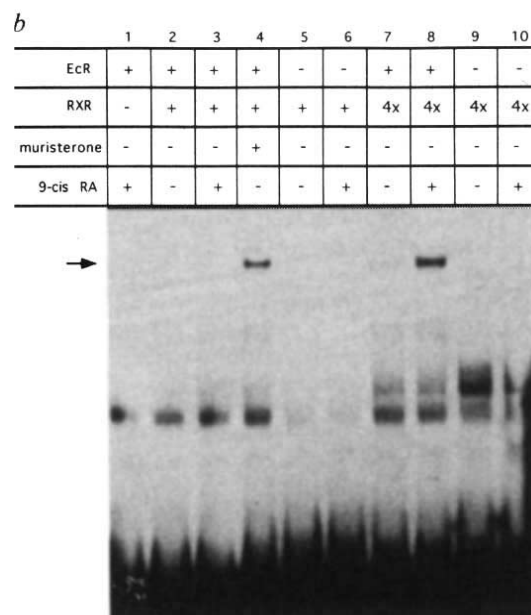


FIG. 3 Muristerone and 9-*cis*-retinoic acid (RA) stimulate EcR-RXR- α but not EcR-USP complex formation. **a** And **b**, RXR- α , EcR and USP were incubated alone or together in the presence of muristerone, 9-*cis* RA, or both, as indicated, and then incubated with wild-type Ec-RE oligonucleotide before gel shift electrophoresis. **b**, As **a**, except that samples in lanes 7-10 contained four times as much RXR- α .

METHODS. As for Figs 1 and 2, except that hormones (10^{-6} M muristerone or 10^{-6} M 9-*cis* RA) were included only where indicated. The quantity of RXR- α used in **a** and lanes 2-6 of **b** was determined by titrating RXR- α against 2 μ l of *in vitro*-translated EcR in the presence of muristerone. We used the minimum input of RXR- α (0.2 μ l) to achieve maximal EcR-RXR- α complex formed on the wild-type Ec-RE oligonucleotide.



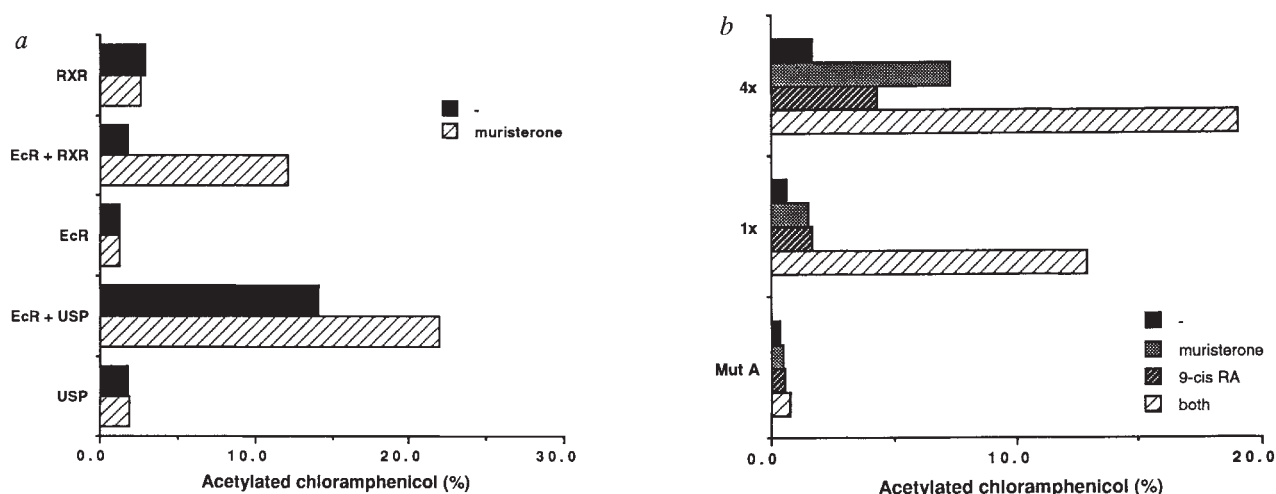


FIG. 4 Transactivation by EcR requires RXR or USP. *a*, CV1 cells were transfected with 4 μ g 4 $\times$ wild-type (wt) Ec-RE CAT reporter plasmid plus 4 μ g each of CMV- β and SG5-RXR- α (RXR); CMV-EcR and SG5-RXR- α (EcR + RXR); CMV-EcR and SG5 (EcR); CMV-EcR and SG5-USP (EcR + USP) or CMV- β and SG5-USP (USP). Cells were treated or not with 10^{-6} M muristerone before assaying for CAT activity. Results shown are the average of 5 independent experiments. *b*, CV1 cells were transfected with 4 μ g CMV-EcR and 4 μ g SG5-RXR- α plus 4 μ g 4 $\times$ wtEc-RE (4 $\times$), 4 μ g 1 $\times$ wtEc-RE (1 $\times$) or 4 μ g Mut A CAT reporter plasmids as indicated, and treated with neither, either or both of 10^{-6} M muristerone, 10^{-6} M 9-*cis* RA before assaying for CAT activity. Results shown are the average of three independent experiments.

METHODS. The Ec-RE CAT reporter plasmids were constructed by inserting

the following oligonucleotides into the *Xba*I site of P50Hsp70 CAT²⁶. 4 $\times$ wt contains two copies of the oligonucleotide CTAGCAGGGTTCAATGCACT-CTAGAAGGGTTCAATGCACTCTAG; Mut A contains one copy of the oligonucleotide CTAGCAGTGCACAATGCACTCTAGAAGTGCACAATGCACTCTAG; 1 $\times$ wt contains one copy of the oligonucleotide CTAGCAGGGTTCAATGCACT-CTAG. Transfections were done using a standard calcium phosphate protocol²⁷. Hormone treatments were for 24 h before harvesting cells. In agreement with ref. 28, we found that several ecdysteroids, including 20-hydroxy-ecdysone, fail to stimulate CAT expression in cotransfer assays with EcR in mammalian cell lines when used at concentrations up to 10^{-5} M. As well as muristerone A (Sigma), effective ecdysteroids in mammalian cells include ponasterone A and the non-steroidal ecdysone RH5849 (data not shown). SG5 plasmids used the SG5 expression vector²⁹.

(Fig. 1*b*, lanes 13–15). This demonstrates that in S2 extracts the activity that EcR needs for binding is limiting and exchangeable. The gel shift complexes were specific for EcR binding, as determined by competition with wild-type and mutated Ec-REs, and because no complex formed on a mutated Ec-RE (Fig. 1*c*, lanes 1–8). The EcR-RXR- α and the transfected HeLa EcR complexes were both quantitatively retarded by an RXR antibody in gel shift experiments (data not shown). These results demonstrate that EcR has little affinity for this Ec-RE without a heterodimer partner such as RXR- α .

The *Drosophila* homologue of RXR is *ultraspiracle*⁶. Although *in vitro*-translated USP had no detectable affinity for the Ec-RE oligonucleotide, mixing USP with EcR produced a complex that comigrated with *Drosophila* S2 activity (Fig. 2, lanes 2 and 3). As EcR heterodimerizes with RXR or USP, then USP may heterodimerize with other partners of RXR. To test this, we mixed USP with RAR- α . Band-shift activity was not detected on the Ec-RE oligonucleotide (data not shown), but was when a retinoic acid response element was used (Fig. 2, lanes 5–8).

These gel shifts with EcR were also done in the presence of muristerone. To test how hormone affects EcR-RXR- α and EcR-USP complexes, we added muristerone, 9-*cis*-retinoic acid, or both to the binding reactions. Figure 3*a* shows that whereas the EcR-USP complex did not require and was not affected by hormone addition (lanes 13–16), the EcR-RXR- α complex was enhanced by either ligand (lanes 5–8). The experiment shown in Fig. 3*a* was done with limiting amounts of RXR- α . When four times more RXR- α was added, 9-*cis*-retinoic acid stimulated complex formation more dramatically (Fig. 3*b*, lanes 7 and 8). No binding of RXR- α to the Ec-RE was observed with or without 9-*cis*-retinoic acid in the absence of EcR (Fig. 3*b*, lanes 9 and 10). The promotion of heterodimer DNA-binding activity by hormone observed here is distinct from previous studies in which hormone alters homodimer DNA-binding activity, destabilizes TR-RAR heterodimers, or induces altered

mobility in gel shift experiments of DNA-bound homodimers (refs 19 and 20, and refs therein).

To test our *in vitro* results functionally, CV1 cells were transfected with EcR and a chloramphenicol acetyltransferase (CAT) gene reporter plasmid containing four Ec-REs with or without cotransfected RXR- α or USP (Fig. 4*a*). Muristerone did not affect CAT expression unless EcR was cotransfected with RXR- α or USP. EcR-USP cotransfection significantly enhanced CAT expression, and muristerone only marginally increased it. The hormone-independent feature of EcR-USP CAT expression, and the marginal increase with muristerone, persisted to the lowest limits of input expression plasmids permitted by assay sensitivity; it was also observed when a reporter plasmid containing a single Ec-RE was used (data not shown).

To corroborate further the functional interaction between EcR and RXR- α , we examined 9-*cis*-retinoic acid effects on CAT expression after cotransfer of EcR and RXR- α . As with muristerone, 9-*cis*-retinoic acid alone stimulated CAT expression when a tetramer of Ec-REs was used as the promoter of the CAT gene (Fig. 4*b*; 4 $\times$). When both hormones were added together, the response was more than their sum effects and was synergistic when the reporter plasmid contained only one Ec-RE (Fig. 4*b*; 1 $\times$). A functional Ec-RE was required, because mutated response elements in the reporter gene promoter failed to mediate a response to hormone (Fig. 4*b*; Mut A).

Our results have demonstrated EcR-RXR- α heterodimerization in a functional assay. We conclude that EcR must heterodimerize for DNA binding and transactivation to be detectable, and that USP or its vertebrate homologue, RXR, can be the partner. The reliance of EcR on USP for its activity in flies is in accordance with the *usp*-phenotype^{21,22}. Our results raise the possibilities that control of EcR activity could include regulation or replacement of the heterodimer partner and that hormone may be important in promoting partner selection. □

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- Clever, U. & Karlson, K. *Expl Cell Res.* **20**, 623–626 (1960).
- Clever, U. *Science* **146**, 794–795 (1964).
- Ashburner, M. *Cell* **61**, 1–3 (1990).
- Koelle, M. R. *et al. Cell* **67**, 59–77 (1991).
- Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. & Evans, R. M. *Nature* **345**, 224–229 (1990).
- Oro, A. E., McKeown, M. & Evans, R. M. *Nature* **347**, 298–301 (1990).
- Yu, V. C. *Cell et al.* **67**, 1251–1266 (1991).
- Leid, M. *et al. Cell* **68**, 377–395 (1992).
- Zhang, X.-k., Hoffmann, B., Tran, P. B.-V., Graupner, G. & Pfahl, M. *Nature* **355**, 441–445 (1992).
- Kliwer, S. A., Umenson, K., Mangelsdorf, D. J. & Evans, R. M. *Nature* **355**, 446–449 (1992).
- Bugge, T. H., Pohl, J., Lonnoy, O. & Stunnenberg, H. G. *EMBO J.* **11**, 1409–1418 (1992).
- Marks, M. S. *et al. EMBO J.* **11**, 1419–1435 (1992).
- Riddihough, G. & Pelham, H. R. B. *EMBO J.* **6**, 3729–3734 (1987).
- Martinez, E., Givel, F. & Wahli, W. *EMBO J.* **10**, 263–268 (1991).
- Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. *Nature* **330**, 444–450 (1987).
- Giguere, V., Ong, E. S., Segui, P. & Evans, R. M. *Nature* **330**, 624–629 (1987).
- Sap, J. *et al. Nature* **340**, 242–244 (1989).
- Wang, H. *et al. Nature* **340**, 163–166 (1989).
- Zhang, X.-k. *et al. Nature* **358**, 587–593 (1992).
- Andersson, M. L. *et al. Nucleic Acids Res.* **20**, 4803–4810 (1992).
- Perrimon, N., Engstrom, L. & Mahowald, A. P. *Genetics* **111**, 23–41 (1985).
- Oro, A. E., McKeown, M. & Evans, R. M. *Development* **115**, 449–462 (1992).
- Schöler, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N. & Gruss, P. *EMBO J.* **8**, 2543–2550 (1989).
- MacGregor, G. R. & Caskey, T. C. *Nucleic Acids Res.* **17**, 2365 (1989).
- Frank, A. *et al. EMBO J.* **11**, 2941–2950 (1992).
- Amin, J., Mestral, R. & Voellmy, R. *Molec. cell. Biol.* **11**, 5937–5944 (1991).
- Chen, C. & Okayama, H. *Molec. cell. Biol.* **7**, 2745–2752 (1987).
- Christopherson, K. S., Mark, M. R., Bajaj, V. & Godowski, P. J. *Proc. natn. Acad. Sci. U.S.A.* **89**, 6314–6318 (1992).
- Green, S., Isseman, I. & Sheer, E. *Nucleic Acids Res.* **16**, 369 (1988).

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TFIIIC relieves repression of U6 snRNA transcription by chromatin

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THE U6 small nuclear (sn)RNA gene (*SNR6*) from the yeast *Saccharomyces cerevisiae* is transcribed by RNA polymerase III *in vivo*¹. This gene is unusual in having a TATA box at position –30, and an essential B-block element located downstream of the T-rich termination signal^{2,3}. The B block is one of the two intragenic promoter elements of transfer RNA genes that are recognized by transcription factor (TF)IIIC (ref. 4). But accurate *in vitro* transcription of yeast U6 snRNA gene by PolIII in a purified system requires only TFIIB components, including the TATA-box binding protein TBP⁵. Here we report that, after nucleosome reconstitution or chromatin assembly, U6 snRNA synthesis becomes dependent on TFIIC and on the integrity of the B-block element. This observation resolves an apparent paradox between *in vitro* and *in vivo* results concerning the necessity of the downstream B-block element^{3,5} and sheds light on a new role of TFIIC in gene activation.

Addition of TFIIC to crude yeast extracts specifically stimulated transcription of the *SNR6* gene when using pB6 plasmid DNA, which harbours the *SNR6* gene with the B block, but not with pTa6 DNA lacking the B block (Fig. 1). As several studies indicated that nucleosomal organization or higher order chromatin structures can act like general repressors of RNA polymerase III (polIII) transcription^{4,6,7}, we postulated that the

requirement for TFIIC could be related to chromatin repression rather than to transcription complex assembly. We have reconstituted nucleosomes with purified histones^{8,9} on pB6 DNA (Fig. 2). At a close to physiological nucleosome density (a mass ratio of core histones to DNA of 0.8 to 1.0 (ref. 10), transcription of the *SNR6* gene was severely inhibited (Fig. 2b, lanes 1–4). But addition of TFIIC after nucleosome formation alleviated this inhibition, though it did not restore control levels (Fig. 2b, lanes 5–8). This antirepression effect was also observed under the same conditions with a tRNA^{Glu} gene, but not with *SNR6* template lacking the B block (pTa6) (results not shown).

Disruption of the B block by a 2-base-pair (bp) deletion ($\Delta 238/9$) inhibits *SNR6* gene transcription in crude extracts and causes cellular lethality³. We analysed the effect of the same mutation on TFIIC–DNA binding using the mobility shift assay (Fig. 3a). A DNA fragment from pB6, encompassing the B block, bound TFIIC (lane 2), whereas the corresponding fragment from mutant pB6 $\Delta 238/9$ was unable to do so (lane 6). In addition, pB6 $\Delta 238/9$ DNA could not compete for TFIIC binding (compare lanes 2 and 4). Remarkably, this B-block mutation completely prevented the enhancement of transcriptional activity by TFIIC after nucleosome reconstitution with purified histones (result not shown). But in the absence of histones, both plasmid DNAs were transcribed with the same efficiency in a reconstituted transcription system, supplemented or not with TFIIC (Fig. 3b, lanes 7–10).

Incubation of plasmid DNAs in a *Xenopus* egg extract induces the assembly of physiologically regularly spaced nucleosomes^{11,12}, which is not the case with nucleosomes reconstituted with histones. *SNR6* templates, pB6 and pB6 $\Delta 238/9$ DNAs,

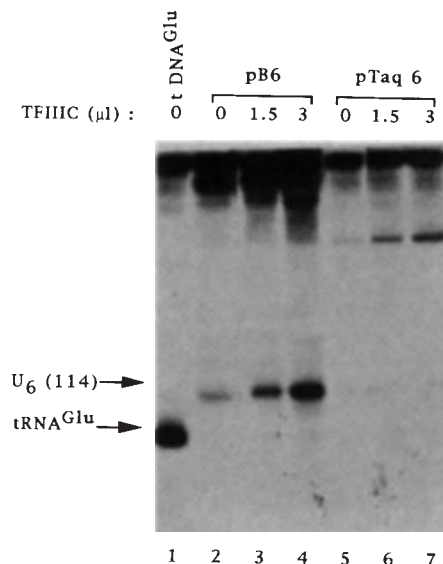


FIG. 1 TFIIC is required for *in vitro* transcription of the *SNR6* gene in a crude yeast extract. Different templates were transcribed in a crude yeast extract, supplemented or not with affinity-purified TFIIC¹⁹. Lane 1: tDNA^{Glu} gene. Lanes 2–4: pB6 DNA. Lanes 5–7: pTa6 DNA (no B block³). Lanes 1, 2 and 5 received 0 ng, lanes 3 and 6, 15 ng, and lanes 4 and 7, 30 ng of TFIIC. The 114-nucleotide (nt) U6 RNA is transcribed only from the B block containing template (pB6 DNA). The TFIIC-dependent transcript in lanes 5–7 is due to a B block located on the plasmid vector.

METHODS. Plasmid pB6 contains a 454-bp DNA fragment encompassing the *SNR6* gene, from positions –140 to +314, amplified by PCR from yeast genomic DNA, and cloned into the *EcoRI* and *BamHI* sites of the Bluescript plasmid (Stratagene). A yeast S100 whole-cell extract was applied to a DEAE–Sephadex column equilibrated with 90 mM KCl (see ref. 20), and 23 μ g of protein from the flow-through fraction were used for *in vitro* transcription of 0.15 μ g DNA. Transcription reactions and electrophoretic analysis of the transcripts were previously described⁵.

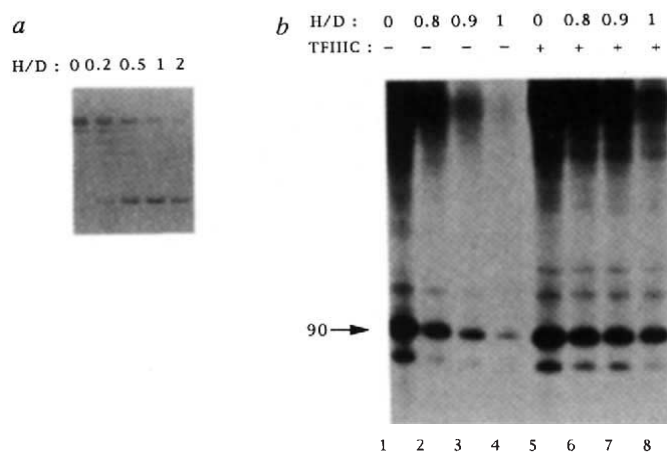


FIG. 2 TFIIC alleviates repression by purified histones. **a**, pB6 DNA supercoiling after nucleosome reconstitution with purified histones, at varying histones-to-DNA ratio (H/D), as indicated. **b**, *In vitro* transcription of nucleosome reconstituted pB6 DNA template, in the absence (lanes 1–4), or in the presence of 20 ng of purified TFIIC (lanes 5–8). Note that the 90-nt transcript is generated from the full-length 114-nt U6 RNA transcript by a specific nuclease present in the Cibacron blue fraction⁵.

METHODS. Nucleosomes were reconstituted onto 0.8 μ g pB6 DNA by dialysis from high salt, using different mass ratios of purified duck erythrocyte histones (H2A, H2B, H3, H4) to DNA (H/D), as described⁹. After 30 min of treatment with 10 units of topoisomerase I at 37 °C, the extent of DNA supercoiling was estimated by electrophoresis on a 0.9% agarose gel at 40 V for 16 h. DNA was then visualized by staining with ethidium bromide. Increasing the mass ratio of core histones to DNA induced progressive supercoiling of plasmid DNA. Micrococcal nuclease digestion of the supercoiled plasmids led to the typical accumulation of a mononucleosome of about 150 bp (result not shown). After nucleosome reconstitution, *in vitro* transcription was done as in Fig. 1, in the presence of 120 mM KCl, 0.1 μ g DNA, 50 ng of pol III, 3.5 μ g of a Cibacron blue fraction containing TFIIB⁵ and, when indicated, 20 ng affinity-purified TFIIC¹⁹.

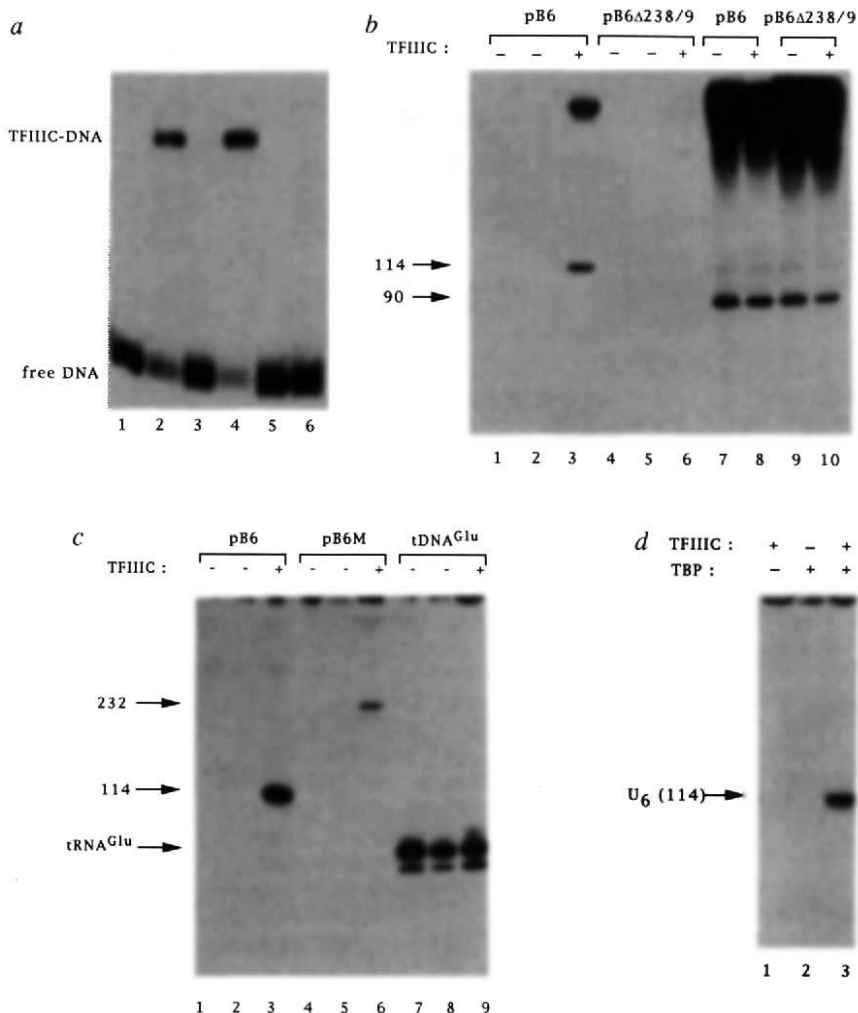
assembled into chromatin in the egg extract, were transcriptionally inert, even when supplemented with polIII and a protein fraction containing TFIIB activity, and thus the TATA-box binding protein (TBP) (Fig. 3b; lanes 1, 2, 4, 5). This effect was observed after 1 h of incubation in the egg extract (Fig. 3b),

or after 4 h, when the extent of chromatin assembly reaches a maximum as judged by a supercoiling assay^{11,12} (data not shown) (Fig. 3c, lanes 1, 2, 4, 5). Under the same conditions, after 4 h of incubation in the egg extract, the yeast tRNA^{Glu} gene was actively transcribed (Fig. 3c, lanes 7–9) in the absence

FIG. 3 An intact B block is necessary for TFIIC binding and transcription of chromatin templates.

a, TFIIC binding to the *SNR6* gene. The 454-bp *EcoRI*–*Bam*HI fragment encompassing the *SNR6* gene from pB6 (lanes 1–4), or from pB6 Δ 238/9 (lanes 5, 6) were used as probes in gel retardation assays using 20 ng affinity-purified TFIIC¹⁹ and different competitor DNAs. Lanes 1 and 5: free DNA probes. Lanes 2 and 6: pBR322 DNA (100 ng) as competitor. Lane 3: 60 ng pB6 DNA, and 40 ng pBR322 DNA. Lane 4: 60 ng pB6 Δ 238/9 DNA, and 40 ng pBR322 DNA. **b**, Mutation of the B block inactivates chromatin templates. *In vitro* transcription of plasmid pB6 (lanes 1, 2, 3, 7 and 8) or pB6 Δ 238/9 (lanes 4, 5, 6, 9 and 10) after 1 h of chromatin assembly (lanes 1–8) or as naked DNA (lanes 9–10). Transcriptions were done without addition of yeast proteins (lanes 1, 4), or in the presence of 50 ng of pol III and 3.5 μ g of Cibacron blue fraction⁵ containing TFIIB (lanes 2, 3, 5, 6). In lanes 3 and 6, 30 ng of purified yeast TFIIC was added just before the *Xenopus* egg extract. The 114-nt and 90-nt U6 RNA transcripts are shown. **c**, Transcription of plasmid PUC-Glu (tDNA^{Glu}), pB6 or pB6M DNA (pB6M harbours a *SNR6* maxigene) after 4 h of chromatin assembly. Chromatin templates were transcribed without addition of yeast proteins (lanes 1, 4, 7), or in the presence of 50 ng of pol III and 1.8 μ g Cibacron blue fraction⁵ containing TFIIB (lanes 2, 3, 5, 6, 8 and 9). Affinity-purified TFIIC was added together with the transcription mixture in lanes 3, 6 and 9. The 114- and 232-nt U6 RNA transcripts and the 90-nt tRNA^{Glu} transcript are shown. **d**, Both TBP and TFIIC are required for transcription of a chromatin template. After 1 h of chromatin assembly, transcription of pB6 DNA was done in the presence of TFIIB* (depleted of TBP)¹⁴ (1.4 μ g protein) with or without TBP (60 ng) or purified TFIIC (30 ng), as indicated. The 114-nt U6 RNA transcript is shown.

METHODS. pB6 Δ 238/9 was constructed by site-directed mutagenesis of the B block of pB6, as in ref. 3. The maxigene version of *SNR6*, in plasmid pB6M, was obtained by a tandem insertion of a 59 bp *XbaI*–*HincII* DNA fragment from Bluescript plasmid within the *EcoNI* site of the *SNR6* gene. This generates a 232-nt U6 RNA transcript. TFIIC–DNA complexes were analysed by gel electrophoresis as described previously¹⁹. Plasmid DNAs (0.15 μ g) were assembled into chromatin by incubation at 22 °C with 10 μ l of *Xenopus* egg extract in the presence of 2 mM ATP and 3 mM MgCl₂, as described¹¹. The extent of



chromatin assembly was measured by supercoiling assay and physiological spacing checked by micrococcal nuclease digestion (results not shown). After 1 h or 4 h of chromatin assembly, transcription reactions were done as in Fig. 1. Separation of TBP from TFIIB was on a Q-Sepharose column, as described¹⁴.

of yeast proteins. This observation is in agreement with a report that a transcriptionally active tRNA gene is not incorporated into a nucleosome *in vivo*¹³. Addition of TFIIC restored transcription of *SNR6* templates having an intact B block, pB6 and pB6M DNA (a maxigene version of *SNR6*) (Fig. 3b, lane 3; Fig. 3c, lanes 3 and 6). Although activation was much less efficient with the stretched gene, increasing the distance between the initiation site and the B-block element did not suppress the ability of TFIIC to stimulate transcription. In contrast, the *SNR6* gene with a mutated B block remained silent (Fig. 3b, lane 6), as well as the truncated gene on pTaq6 DNA (result not shown). TFIIC was able to restore transcription of the B block-containing genes when added after chromatin assembly (Fig. 3c, lanes 3 and 6). Considering a possible heterogeneity in chromatin templates, we have used the purified reconstituted system to check that addition of TFIIC had no stimulatory effect at very low concentrations of pB6 DNA (results not shown). Therefore, TFIIC was likely to act on chromatin templates and not on a limited number of non-nucleosomal DNA molecules. We explored the possibility that TFIIC could substitute for TBP to help assemble TFIIB on the *SNR6* promoter. For this experiment, TFIIB was resolved from TBP by Q-Sepharose chromatography¹⁴. TFIIC and TBP were both required for transcription of the chromatin pB6 template (Fig. 3d).

This study allows us to define a new role for TFIIC in activating chromatin, besides its known function in transcription complex assembly^{15,16}. When the *SNR6* gene is complexed with histones (Fig. 2), or assembled into chromatin in a crude extract where many proteins can interact with DNA (Fig. 3c), TFIIC seems to be required to help transcription factors gain access to DNA and to stabilize productive transcription complexes. How TFIIC fulfills this function is not clear, but it involves its binding to the downstream B block. Binding of TFIIC to the B block could perturb chromatin structure, allowing assembly of TFIIB components at the upstream promoter. Transcription complexes could be stabilized by further interaction with TFIIC, possibly in synergy with one of the two overlapping pseudo A blocks located close to the start site^{3,17}.

It has been suggested that yeast TFIIC, with its two-domain structure ($M_r \sim 300K$ each), plays the double role of an enhancer binding factor (through B-block interaction) and of an assembly factor (through A-block binding)¹⁸. These functions are assigned to different proteins in class II genes. Thus the mode of activation of class III and class II genes appears increasingly related by the aptitude of a class III factor to activate transcription on a chromatin template from a distant and 3' position, as shown here. □

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1. Moenne, A. et al. *EMBO J.* **9**, 271-277 (1990).
2. Brown, D. A. & Guthrie, C. *Nature* **334**, 213-218 (1988).
3. Brown, D. A. & Guthrie, C. *Genes Dev.* **4**, 1345-1356 (1990).
4. Geiduschek, E. P. & Tocchini-Valentini, G. A. *Rev. Biochem.* **57**, 873-914 (1989).
5. Margottin, F. et al. *Science* **251**, 424-426 (1991).
6. Wolffe, A. P. *Curr. Biol.* **6**, 366-368 (1991).
7. Felsenfeld, G. *Nature* **355**, 219-224 (1992).
8. Stein, A. J. *molec. Biol.* **130**, 103-134 (1979).
9. Zivanovic, Y. et al. *J. molec. Biol.* **214**, 479-495 (1990).
10. Laybourn, P. J. & Kadonaga, J. T. *Science* **254**, 238-245 (1991).
11. Almouzni, G. & Mèchali, M. *EMBO J.* **7**, 4355-4365 (1988).
12. Laskey, R. A., Mills, A. D. & Morris, N. R. *Cell* **10**, 237-243 (1977).
13. Morse, R. H., Roth, S. Y. & Simpson, R. T. *Molec. cell. Biol.* **12**, 4015-4025 (1992).
14. Huet, J. & Sentenac, A. *Nucleic Acids Res.* **20**, 6451-6454 (1992).
15. Lassar, A. B., Martin, P. L. & Roeder, R. G. *Science* **222**, 740-748 (1983).
16. Kassavetis, G. A., Braun, B. R., Nguyen, L. H. & Geiduschek, E. P. *Cell* **60**, 235-245 (1990).
17. Bordonné, R. & Guthrie, C. *Nucleic Acids Res.* **20**, 479-485 (1992).
18. Gabrielsen, O. S. & Sentenac, A. *Trends Biochem. Sci.* **16**, 412-416 (1991).
19. Gabrielsen, O. S., Marzouki, N., Ruet, A., Sentenac, A. & Fromageot, P. *J. biol. Chem.* **264**, 7505-7511 (1989).
20. Riggs, D. L. & Nomura, M. *J. biol. Chem.* **265**, 7596-7603 (1990).

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Articles are research reports whose conclusions are of general interest and which are sufficiently rounded to be a substantial advance in understanding. They should not have more than 3,000 words of text (not including figure legends) or more than six display items and should not occupy more than five pages of *Nature*.

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Protection and susceptibility

The association between insulin-dependent diabetes mellitus and HLA is fearsomely complicated. But powerful techniques are being brought to bear on the problem.

THERE have been a few successes in unravelling the genetic basis of diabetes, notably the discovery of mutations in the glucokinase gene which underlie a form of non-insulin dependent (type 2) diabetes mellitus (NIDDM), called maturity-onset diabetes of the young¹⁻⁴. But in general identifying the genetic contribution to diabetes is a daunting task, not only for NIDDM but also for the less common insulin-dependent (type 1) diabetes mellitus (IDDM).

In contrast to the simple mendelian pattern of inheritance that underlies maturity-onset diabetes, IDDM results from the complex interaction of a number of genetic and environmental factors⁵. Together, these factors conspire to launch a T-cell-mediated autoimmune attack on the pancreatic β -cells which normally secrete insulin. Of the genetic factors, there is good evidence that the region surrounding the insulin gene on chromosome 11 plays a part⁶ — indeed new studies show that this susceptibility region is confined to a 4.1-kilobase stretch of DNA encompassing the insulin gene⁷. Recent work has also incriminated the immunoglobulin heavy chain region⁸. But without doubt the single biggest genetic factor that remains to be understood is the contribution of the HLA region on chromosome 6, and a report in this month's *Nature Genetics* may pave the way towards this goal⁹.

The major histocompatibility complex spans about four megabases and the HLA-D genes, which code for the class II glycoproteins, are spread across a good 20 per cent of that length¹⁰. The class II molecules (HLA-DR, -DQ and -DP) are heterodimeric membrane proteins consisting of an A and B chain involved in antigen presentation. A large body of evidence suggests that the class II genes, and especially certain alleles of the DR and DQ loci, confer susceptibility to IDDM⁵. This certainly applies to alleles at the HLA-DRB1 locus: HLA-DR3 and -DR4 are positively associated with IDDM, whereas HLA-DR2 is nega-

tively associated with the disease.

There is also considerable evidence that the DQ loci have an important role in determining risk or susceptibility. The best known example is the residue at position 57 of the HLA-DQB1 gene. Many studies have indicated that aspartate 57 confers protection to IDDM, and this is supported by the fact that the non-obese diabetic (NOD) mouse — a model for IDDM — has a serine at residue 57 in the homologous gene product while non-diabetic strains have aspartate. But although this residue is important, it does not tell the whole story. And because of the considerable linkage disequilibrium observed across the HLA region, there are many genes which may actually be responsible for controlling IDDM risk.

In an attempt to understand the relative roles of the HLA-DR and -DQ genes, Henry Erlich and his colleagues⁹ have performed a detailed molecular analysis of IDDM using HLA typing based on the polymerase chain reaction. By studying the precise DNA sequence of these key HLA alleles, Erlich and colleagues say they can extract much more information (on subtypes of particular alleles, for example) than if they were to rely on the more common serological analysis alone.

Erlich's group examined more than 250 members from affected Mexican-American families — descendants of Hispanic Caucasians and native Americans. Because of the very low incidence of IDDM among native Americans, the Mexican-American population also has a lower incidence of IDDM than Caucasians. Two alleles of the DRB1 locus — DR4 and especially DR3 — cropped up much more often in patients than controls, as largely expected from previous studies. The authors also found that a DR3/DR4 heterozygous genotype appears to confer a greater risk for IDDM than homozygosity for either DR3 or DR4 (as seen in previous studies), meaning that these two gene products may interact synergistically.

There are also some 'protective' haplotypes, however, which are significantly less common among IDDM patients. For example, some DR2 haplotypes (such as DRB1*1501,DQB1*0602) are protective. In contrast, the DR2 haplotype DRB1*1602,DQB1*0301, frequently found in native Americans but

not Caucasians, is not. Erlich *et al.* found that all the high-risk haplotypes are of European origin, whereas the most protective haplotype (DRB1*1402) is of native American origin, largely accounting for the differences in IDDM susceptibility among these populations. (Environmental differences may also exist, of course.)

This analysis also reveals the specific role of the DRB1 locus within the DR4 haplotype in influencing IDDM risk. Virtually all of the Mexican-American DR4+ patients have the DQB1*0302 allele, which is non-Asp 57. However, variation at DRB1 (nine different alleles were detected) seems to confer varying degrees of risk. The difference between two of these alleles — DRB1*0405 and DRB1*0408 — lies solely at residue 57, with serine in that position in the high-risk group but aspartate in the low risk. However, Ser 57 alone does not predict high IDDM risk, and nor does Asp 57 always confer protection.

Perhaps most notably, the typing methods employed by Erlich *et al.* allowed a detailed examination of the combined effects of specific DR-DQ haplotypes, including the significance of residue 57 of DQB1. The same DQB1 allele (encoding Asp 57) is present on both DRB1*1402 and DRB1*1602 haplotypes, yet these are highly protective and neutral, respectively. So although Asp 57 in DQB1 is clearly important in conferring resistance to IDDM — it is present in all protective haplotypes — it is not sufficient to assure protection.

So progress is being made in getting to grips with IDDM. But it will take a great deal more work in humans, as well as in valuable animal models such as the NOD mouse and the BB rat¹¹, before this classic polygenic disorder can be said to be even partly understood.

Kevin Davies

Kevin Davies is Editor of Nature Genetics.

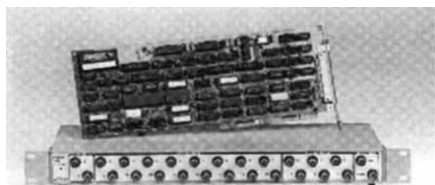
1. Vionnet, N. *et al.* *Nature* **356**, 721–722 (1992).
2. Stoffel, M. *et al.* *Nature Genet.* **2**, 153–156 (1992).
3. Stoffel, M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 7698–7702 (1992).
4. Froguel, P. *et al.* *New Engl. J. Med.* **328**, 697–702 (1993).
5. Todd, J. *Immun. Today* **11**, 122–129 (1991).
6. Bain, S. C. *et al.* *Nature Genet.* **2**, 212–215 (1992).
7. Lucassen, A. M. *et al.* *Nature Genet.* (in the press).
8. Field, L. L. *et al.* *Am. J. hum. Genet.* **49**, 627–634 (1991).
9. Erlich, H. E. *et al.* *Nature Genet.* **3**, 358–364 (1993).
10. Trowsdale, J., Ragoussis, J. & Campbell, R. D. *Immun. Today* **12**, 443–446 (1991).
11. Jacob, H. J. *et al.* *Nature Genet.* **2**, 56–60 (1992).

Also in this month's *Nature Genetics*: the molecular basis of Thomsen's disease; the channel activity of purified $\Delta F508$ CFTR; localization of the gene for familial juvenile nephronophthisis; and a review article from Ahn and Kunkel on the structural and functional diversity of dystrophin.

Californian sampler

This week's crop of new products from manufacturers in California includes a data acquisition system for the personal computer, an assortment of monoclonal antibodies and a UV-visible spectroscopy system.

AXON Instruments announces the release of the Digidata 1200 **data acquisition system** for IBM PC AT-compatible computers (*Reader Service No. 100*). The system is designed for low-noise experiments, such as physiological recordings, and provides acquisition, stimulation and environmental control functions. It features 32 12-bit analog-to-digital input channels with ± 10 -V range, a 330 kHz sampling rate, two 12-bit digital-to-analog output channels, eight digital inputs and eight digital outputs. The complete system consists of a plug-in board, a shielded cable and an interface box. Optional



PC-based data acquisition system.

interfaces are available. The Digidata 1200 includes a range of features, including programmable input gain from one to eight for a maximum resolution of 625 μ V per A/D unit; channels that can be sampled in random order, with a differential gain applied to each channel; the ability to transfer simultaneously D/A and A/D data on two DMA channels for precise synchronization; and a five-channel counter and 48-bit real-time clock incorporated into the design. The device is supported by the company's pCLAMP, AxoTape and AxoBASIC acquisition software.

■ Monoclonal marketplace

Dako has introduced a new **monoclonal B-lymphocyte antibody**, BLA.36, that not only recognizes haematopoietic cell antigens in paraffin-embedded material but also identifies reactive and malignant B cells, Reed-Sternberg cells and all four variant cell subtypes of Hodgkin's disease (*Reader Service No. 101*). It does not label T lymphocytes. In addition, Dako says that comparison studies have also indicated that anti-BLA.36 labels Hodgkin's disease more consistently than LeuM1, Ber-H2, L26, UCHL-1 and LN1 (Della Croce, D. R., Iman, A., Brynes, R. K., Nathwani, B. N. & Taylor, C. R. *Haem. Oncol.* 9, 103 (1991)). Studies conducted on normal lymphoid tissue with BLA.36 demonstrated staining of most small and large noncleaved cells and immunoblasts, and a small minority of small cleaved cells. No labelling of macrophages was

observed. Further analysis on several Hodgkin's cases identified classical Reed-Sternberg cells and variants including mononuclear Hodgkin's cells, L and H (lymphocytic and histiocytic variant) cells of the lymphocyte predominant type, lacunar cells in nodular sclerosis, pleomorphic depletion and necrobiotic 'mummy' cells.

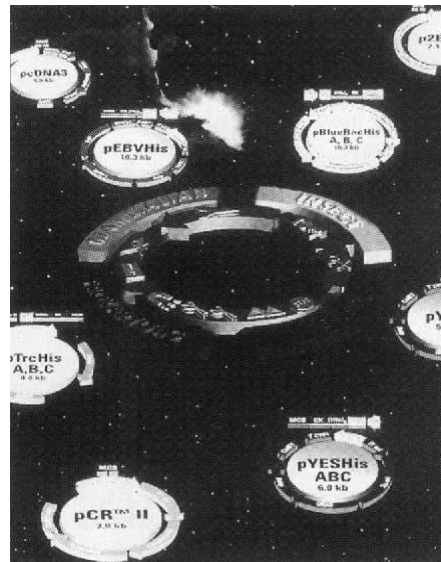
BaBCO supplies a **monoclonal antibody specific for mannosidase II** — a 135-kD Golgi membrane enzyme that acts as a protein marker for the Golgi apparatus (*Reader Service No. 102*). Stated applications of the monoclonal antibody include immunofluorescence, immunoprecipitation, immunoblotting, immunolabelling and other immunocytochemical studies. It is available as ascites fluid.

Zymed offers a new **mouse monoclonal antibody to CD45RO** that has affinity to an epitope that is strongly expressed on the lower-molecular-weight isoform (p180) of the human leukocyte common antigen (LCA) family and weakly expressed on 190 (exon B) and 205 (exon AB) isoforms of LCA (*Reader Service No. 103*). The antibody reacts with an epitope that is closely related to the UCHL1 epitope. However, Zymed says that its antibody has been shown to be significantly more sensitive than UCHL1 antibody in immunohistostaining formalin-fixed, paraffin-embedded tissues. The antibody is available in purified form for immunohistology. F(ab')₂ fragments are available conjugated to fluorescein isothiocyanate, phycoerythrin and biotin for use in flow cytometry.

Anti-phosphotyrosine monoclonal antibody is Kamiya Biomedical's mouse monoclonal IgG1 antibody, which is prepared by the fusion of mouse myeloma P3/X63-Ag8 cells and phosphotyrosine-KLH immunized spleen cells from a BALB/c mouse (*Reader Service No. 104*). The monoclonal antibody has specific reactivity with phosphorylated growth factor receptors and shows no crossreactivity with phosphoserine and phosphothreonine, Kamiya says. It should be a useful tool for analysing the signal transduction pathway of growth factors in order to explain the higher proliferative response of tumour cells.

■ Molecular research tools

Invitrogen announces the availability of over 40 different **prokaryotic, mammalian, insect and yeast vectors** (*Reader Service No. 105*). A wide variety of these vectors are



Vector varieties — see Invitrogen.

available for *Escherichia coli*, mammalian, yeast and Baculovirus expression and cloning systems. The company also offers a free vector poster providing complete maps and details of the vectors.

A host of new products for molecular biology research are described in the new 1993 Stratagene **catalogue** (*Reader Service No. 106*). The application-orientated catalogue covers cloning vectors, gene libraries and their construction, DNA transfer systems, DNA restriction and modifying enzymes, nucleic acid purification systems, cell biology products, equipment for electrophoresis, nucleic acid transfer and hybridization, image analysis, temperature cycling, and labware products. It also contains an appendix including vector maps and tables with background information.

■ Detection systems

BioGenex Laboratories has two new **western blot test kits** that are designed to provide a confirmation of antibodies to human immunodeficiency virus-1 (HIV-1) and human T-lymphotrophic virus-1 (HTLV-1) in serum and plasma in 2 h (*Reader Service*



HIV-1 and HTLV-1 western blot kits.

No. 107). The kits contain all the necessary ready-to-use reagents, disposable incubation trays, control sera, instruction manual and record forms. Predeveloped reference strips are also included to simplify band identification. The kits can be developed manually or with the company's MAPS (membrane assay processing system) processors — instruments that are intended to simplify and expedite western blot processing, ensuring standardized results and facilitating safe waste disposal.

The new Vector VIP **peroxidase substrate** produces an intense violet-coloured permanent precipitate using the Vectastain Elite ABC kit or other peroxidase-based detection systems in immunohistochemistry (*Reader Service No. 108*). Like DAB, the reaction product is insoluble in alcohols and clearing agents, and so permanently-mounted sections can be prepared using this substrate. However, unlike DAB, Vector says that the Vector VIP substrate does not appear to stain red blood cells in tissue sections. This substrate can be used alone, or in conjunction with DAB or other substrates, in multiple labelling protocols. The high contrast with the DAB colour makes Vector VIP substrate a suitable companion for double staining methods. It is also useful for developing transfer/dot blots on nitrocellulose, nylon or PVDF membranes, producing a reaction product that does not fade at the site of the antigen, DNA or RNA target. Vector's substrate kit consists of four concentrated stock solutions packaged in convenient dropper bottles. Each kit contains sufficient reagents to stain about 2,000 tissue sections or over 150 100-cm² blots. Development times range between 2 and 15 min.

■ HPLC

With the new Beckman 125 **binary gradient pump** for the System Gold family of HPLC modules, binary gradients are formed by two liquid heads, each using patented single-piston, rapid-refill technology (*Reader Service No. 109*). Beckman says that this set-up provides reproducibility, at better than 0.1 per cent, throughout the entire flow-rate range of the pump. A feedback system that monitors the brief rapid-refill cycle and

compensates for the flow deficit is also incorporated in the pump — a combination designed to ensure accurate and reproducible flow under any solvent conditions. The Beckman 125 pump can accommodate microbore to semipreparative applications. The standard configuration provides flow rates to 10 ml min⁻¹; the preparative conversion kit extends this to 30 ml min⁻¹.

The new **flow-through conductivity monitor** (model ICM-180) from Lazar Research Laboratories can be used to optimize the separation and recovery of proteins using both column and HPLC systems (*Reader Service No. 110*). The conductivity cell attaches to the eluent end of the column and digitally displays conductivity during a run. Protein tracking can be accomplished by monitoring eluent conductivity during column separations, as a protein will usually elute from the column at the same conductivity value run after run, Lazar says. The conductivity cell offers a dead volume of less than 20 µl, ensuring no remixing of the separated protein components. The cell is also biocompatible and will resist protein binding to the internal sensor. The monitor has a broad conductivity range to accommodate most salt gradients and attaches directly to strip-chart recorders.

■ Made to measure

Labindustries' newest addition to its positive displacement, zero carryover **micropipettor line**, the P-515 Poppette micropipettor, will sample and deliver 0.5 to 15 µl with



Positive displacement micropipettors.

presterilized, preassembled, disposable plunger and tip sets (*Reader Service No. 111*). The company says that the line offers the advantages of positive displacement — the delivery of precise volumes regardless of surface tension, vapour pressure, density and viscosity — in addition to zero carryover. Two adjustable, precalibrated Poppette micropipettor models are available covering the range 0.5 to 250 µl.

Molecular Bio Products, manufacturer of a line of ART (aerosol-resistant tips) **pipette tips** introduces its two newest tips for autoclavable Eppendorf pipettors (*Reader Service No. 112*). The new 1000 and 250E tips for Eppendorf 200–1,000 µl and the 50–250 µl pipettors eliminate cross-contamination, MBP says.

■ Analytical instrumentation

The new HP8452Win diode-array, UV-visible **spectroscopy system** from Hewlett-Packard, which runs under Microsoft Windows, is designed for two main analytical tasks: the development of methods and research requiring comprehensive spectral manipulation, data evaluation and quantification, and routine testing that requires automated procedures and compliance with good laboratory practice requirements (*Reader Service No. 113*). Up to three confirmation-analysis methods can be used in addition to the main analytical method to



UV-visible spectroscopy system.

check the identity of samples and detect impurities and other sources of error. The statistical tools and method development tasks include: correlation coefficient and other statistical values (evaluates the quality of single component calibration curves), evaluate standards (indicates which wavelengths provide the greatest calibration precision), and optimize wavelength (indicates which wavelengths give the best quantification accuracy). The HP8452Win system is controlled by a UV-visible HP ChemStation that provides data management, system control and networking.

Last month, Varian introduced the UltraMass computer-controlled **inductively coupled plasma-mass spectrometer** (ICP-MS) (*Reader Service No. 114*). UltraMass permits a variety of tasks to be accomplished simultaneously, even while an automated run is taking place. And, as a way of enhancing productivity, the system can be used to perform unattended, overnight analysis. Design features of UltraMass include computer control and storage of all operating parameters in a method, enabling a variety of sample matrices to run without stopping; placement of the sample introduction system outside the instrument to avoid temperature changes from the plasma (previously a major source of drift in analytical signal); and a safety device that measures large peaks without damage to the detector. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.



Pump it up — see Beckman.